Promoters:

Prof. Dr. ir. Stefaan De Smet
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University; Ghent, Belgium

Dr. ir. Noël Dierick
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University; Ghent, Belgium

Prof. Dr. ir. Dirk Fremaut
Department of Animal Production, Faculty of Biosciences and Landscape Architecture, University College Ghent; Ghent, Belgium

Members of the examination committee:

Dr. Jean-Paul Lallès
L'Institut National de la Recherche Agronomique, Systèmes d'Elevage Nutrition Animale et Humaine; Saint Gilles, France

Prof. Dipl.-Ing. Dr. Wilhelm Windisch
Division of Animal Food and Nutrition, Universität für Bodenkultur; Wien, Austria

Prof. Dr. Theo Niewold
Department of Biosystems, Division of Livestock-Nutrition-Quality, Faculty of Bioscience Engineering, Katholieke Universiteit Leuven; Leuven, Belgium

Prof. Dr. ir. Geert Janssens
Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University; Merelbeke, Belgium

Dr. Sam Millet
Institute for Agricultural and Fisheries Research, Animal Sciences Unit; Melle, Belgium

Prof. Dr. ir. Veerle Fievez (Secretary)
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University; Ghent, Belgium

Prof. Dr. ir. Norbert De Kimpe (Chairman)
Department of Organic Chemistry, Faculty of Bioscience Engineering, Ghent University; Ghent, Belgium

Dean:

Prof. Dr. ir. Guido Van Huylenbroeck

Rector:

Prof. Dr. Paul Van Cauwenberge

JORIS MICHIELS

EFFECT OF ESSENTIAL OILS ON GUT BACTERIA AND FUNCTIONALITY IN THE PIG

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

EFFECT VAN ESSENTIËLE OLIËN OP DE DARMBACTERIËN EN –FUNCTIONALITEIT BIJ HET VARKEN

Part of this work was supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) via the research project entitled 'Reductie van *Salmonella*-uitscheiding bij het varken: aangepaste voederstrategieën primordiaal voor de Vlaamse varkenshouderij (2004-2006)'

This research was performed at the Laboratory for Animal Nutrition and Animal Product Quality of the Ghent University (Lanupro, Melle, Belgium) and at the Department of Animal Production of the University College Ghent (Gent, Belgium).

To refer to this thesis:
Michiels, J. (2009). Effect of essential oils on gut bacteria and functionality in the pig. PhD thesis, Ghent University, Belgium, 282p.

ISBN 978-90-5989-293-4

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine or serotonine
ADFI	average daily feed intake (g/day)
ADG	average daily gain (g/day)
AMGP	antimicrobial growth-promoters
ANE	(*E)*-anethole
AUC	area under the time-response curve
BA50	bactericidal activity 50% (µg/mL)
BHT	butylated hydroxytoluene
BP	British pharmacopoeia
BW	body weight (kg)
C	crypt depth
CaCC	calcium-activated chloride channel
CAEO	cassia essential oil
CAR	carvacrol
CCβ	detection capability (mg/kg or mg/L)
CDi	conjugated diene
CIN	(*E)*-cinnamaldehyde
CEO	clove essential oil
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming units (log_{10}/mL or g)
COX	cyclooxygenase
CV	coefficient of variation (%)
DC	digestible carrier
DGGE	denaturing gradient gel electrophoresis
DHA	docosahexaenoic acid (22:6n-3)
DPPH	diphenylpicryl hydrazyl (radical)
EC	enterochromaffin cells
EC50	half maximum effective concentration (mg/L)
EO	essential oil
EP	European pharmacopoeia

ETEC	enterotoxigenic *Escherichia coli*
EUG	eugenol
F	bioavailability (%)
FCR	feed conversion ratio
FEO	fennel essential oil
GABA	gamma-aminobutyric acid
GALT	gut-associated lymphoid tissue
GIT	gastro-intestinal tract
GLUT	glucose transporter
GSH	reduced glutathione
GST	glutathione S-transferase
ICA	inert carrier
IC50	inhibitory concentration 50% (µmol/L, mg/L or otherwise)
IEL	intra-epithelial lymphocytes
IGF-1	insuline-like growth factor 1
IL-1β	interleukin 1 beta
LC50	lethal concentration 50% (mg/L)
LD50	lethal dose 50% (mg/kg body weight)
LPL	lamina propria lymphocytes
LPS	lipopolysaccharides
Isc	short-circuit current (µA/cm^2)
MBC	minimum bacteridal concentration (µg/mL)
ME	micro-encapsulation
MEC	minimum effect concentration (mg/L)
MIC	minimum inhibitory concentration (µg/mL)
MRL	maximum residue limit (mg/kg)
MSDS	material safety data sheet
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOEC	no observable effect concentration (mg/L)
NOEL	no observable effect level (mg/kg body weight or otherwise)
NOAEL	no observable adverse effect level (mg/kg body weight or otherwise)
OEO	oregano essential oil
PCR	polymerase chain reaction
PD	potential difference (mV)
PE	proliferative enteropathy

PGE_2	prostaglandin E2
PML	polymorphonuclear leukocytes or granulocytes
ROS	reactive oxygen species
RSD	residual standard deviation
Rt	transepithelial resistance ($\Omega.cm^2$)
SBM	soybean meal
SCFA	short chain fatty acids
SE	standard error
SGLT	sodium-glucose linked co-transporter
SI	small intestine
SOD	superoxide dismutase
TBARS	thiobarbituric reactive substances
TEO	thyme essential oil
THY	thymol
TNF	tumor necrosis factor
TRPA	transient receptor potential A
TRPV	transient receptor potential V
UFA	unsaturated fatty acids
V	villus length
VIP	vasoactive intestinal peptide

TABLE OF CONTENTS

General background

Since the discovery and development of the first antibiotics prior to the Second World War, these drugs have played an important role in curing disease in humans and animals. In 1946 experiments showed that low, sub-therapeutic levels of antibiotics could improve feed conversion and growth in animals, and the addition of various antibiotics to feed for livestock was initiated. These **antibiotics, supplemented to the diet at nutritional levels (<100 mg/kg),** were designated as **antimicrobial growth-promoters (AMGP)**, since they improved animal performance. However, they can be considered more to be growth permitting rather than growth promoting in the sense that they can only permit the animal to grow to its genetic potential given the diet it is fed. There has been widespread incorporation of AMGP into animal feeds in many countries (*e.g.* Cromwell, 2002). Feeding pigs with sub-therapeutic levels of antibiotics has been documented to increase weight gain by 3.3–8.8% and improve feed conversion by 2.5–7.0% in a nearly consistent way (Doyle, 2001). The effect of AMGP is most pronounced in young growing animals especially under unfavourable hygienic and management conditions. When animals get older, the beneficial effect is reduced and can often not be observed in the finishing period (Wenk, 2003). At weaning, piglets are exposed to a range of stressors, including changes in social settlement, diet composition, environment and bacterial challenges, contributing to digestive upsets and depressions in growth rate. The AMGP have proven to be very effective in counteracting the negative effects manifested around weaning of pigs (Visek, 1978).

The exact **mechanisms** by which this occurs are not completely understood. Since AMGP provide a relatively greater improvement in farms with poor hygiene, their effectiveness is at least partially due

to suppression of some pathogenic bacteria and related gut infections/growth-depressing toxins. It is also believed that the antibiotics alter the normal, non-pathogenic bacteria of the gut and these changes have a beneficial effect on digestive processes and the utilization of nutrients in feed (reviewed by Anderson *et al.*, 1999; Corpet, 2000; Gaskins *et al.*, 2002 and Visek, 1978), since:

- a reduction and/or metabolic inactivation of the gut bacteria diminishes the competition for (essential) nutrients and losses by conversion of nutrients to components with lower nutritional value (SCFA – short chain fatty acids, lactic acid) in the proximal regions of the gut. It has been estimated that as much as 6% of the net energy in a pig's diet may be lost due to microbial fermentation in the stomach and SI (Vervaeke *et al.*, 1979);
- gut bacteria inactivate pancreatic digestive enzymes and bile salts (deconjugation and dehydroxylation; impairment of lipid digestion and production of toxic degradation products) (Eyssen, 1973) and
- gut bacteria metabolize dietary protein with the production of harmful and growth-depressing deamination metabolites like ammonia, irreversible decarboxylation products (biogenic amines) such as cadaverine (*e.g.* Dierick *et al.* 1986ab) and phenolic compounds such as phenol and *p*-cresol.

Antibiotics inhibit these activities and thus increase the digestibility of dietary nutrients. Most AMGP target G+ bacteria, and it is well established that G+ bacteria are predominant in the SI and are mainly responsible for the above mentioned competitive effects. Antibiotics also appear to prevent irritation of the intestinal lining and may enhance uptake of nutrients from the intestine by thinning of the mucosal layer. A lower turnover rate of enterocytes and a lower stimulation of the GALT (gut-associated lymphoid tissue) (inflammation) has been documented as well. The consequence of this is that more nutrients are available for animal growth and a lower maintenance costs are needed to assure the absorptive and immunological functions of the gut. Dierick *et al.* (2002a) stated that growth promotion by feed antibiotics is related and proportional to the inhibition of the total microbial load in the stomach and the jejunum. A reduction of the general bacterial growth in SI and

pathogen proliferation should be major targets to improve animal performance and/or health (Apajalahti & Kettunen, 2006). The involvement of gut microbiota in the mode of action of AMGP is most well substantiated by studies with germ-free animals, carried out as early as in the 60's. For example, feeding AMGP did not improve animal performance in germ-free animals, while animals infected with gut microbiota from conventional animals resulted in growth-depression. AMGP are able to partially reverse this depression in growth (see review by Anderson *et al.*, 1999). In opposite, others believe that the growth-promoting effect of the AMGP cannot be attributed to an antibiotic effect on the microbes of the GIT (gastro-intestinal tract), but rather to a direct effect on host cells, in particular inflammatory cells (Niewold, 2007). Certain antibiotics can accumulate in inflammatory cells (macrophages and PML, polymorphonuclear leukocytes) and inhibit their function (*e.g.* Schoevers *et al.,* 1999). Niewold (2007) suggests that as a consequence the levels of pro-inflammatory cytokines like IL-1 (interleukin-1) would be lower in AMGP-fed animals as compared to control animals, which would result in a lower catabolic stimulus and appetite depression (Klasing *et al.,* 1987). The release of these cytokines triggers an acute phase response which is associated with metabolic expenses. Furthermore, it has been described that the GIT is in a state of constant controlled inflammation. Hence, it is assumed that AMGP reduce this inflammation and subsequently spare energy for production. Finally, experiments with chlortetracycline, penicillin, and sulfamethazine as growth-promoters have shown that treated pigs have higher serum levels of IGF-1 (insuline-like growth factor 1) (Hathaway *et al.*, 1996). Thus the effects of sub-therapeutic antibiotics may extend beyond digestion in the intestine and stimulate metabolic processes throughout the animal.

The use of AMGP would not have become under debate if antibiotic **resistance** did not show up. As soon as in 1947, penicillin resistance was documented. The presence of an antibiotic may kill most of the bacteria in an environment but the resistant survivors (resistance genes by mutation) can eventually re-establish themselves and pass their resistance genes on to their offspring and, often, to

other bacteria of the population or bacteria of a different species (horizontal transfer of resistance genes). These genes may confer resistance to one or several different antibiotics. Both medical and veterinary uses of antibiotics have resulted in the appearance of resistant strains of bacteria. Resistant bacteria which are human pathogens (*e.g. Campylobacter* spp*., Salmonella* spp.) may cause diseases that are difficult to treat. Even if the resistant bacteria are not human pathogens, they may still be dangerous because they can transfer their resistance genes to other bacteria that are pathogenic. There is strong, but not conclusive evidence that the use of AMGP in livestock has contributed significantly to the selection for antibiotic resistance (*e.g.* Mathew *et al.*, 1998) and that these resistant bacteria have spread from farm animals to human through animal products (zoonotic pathogens) (*e.g.* Seyfarth *et al.*, 1997), compromising treatment of infectious diseases. Although resistance may develop very quickly, nutritional antibiotics, were and are still, where allowed (*e.g.* USA) very efficacious in animal production (Cromwell, 2002). Another concern are possible **residues** of these substances in animal products and the environment. Moreover, the **consumer demand** is for safer food, without reliance on antibiotics as feed additives. These concerns resulted in a number of government initiatives, among them measures to reduce veterinarian prescription of antibiotics (therapeutic use) and measures against use of certain antibiotics as growth-promoters. Already in 1969, The Swann Committee in the UK recommended restrictions in the use of antibiotics for growth promotion in order to mitigate the risk of resistance. In Europe, this led to a progressive withdrawal of several substances from the list of authorised additives because of a decision to restrict certain antibiotics to therapeutic use (Dibner & Richards, 2005). Finally, the **EU decided to ban** the four remaining authorised **AMGP** (avilamycin, flavophospholipidol, monensin and salinomycin) by January 2006 (Regulation 1831/2003, EC). In the rest of the world, AMGP are under debate as well and approvals for their use are disappearing.

It is difficult to evaluate the effect of the withdrawal of AMGP in the EU, since comparable data on animal performances and health before and after the ban are hard to find. However, field data indicate that young pigs still suffer from a range of gastro-intestinal disorders (*e.g.* post-weaning diarrhoea), that hereby 'theurapeutic' antibiotics (*e.g.* colistin) are widely used and that some 'old' pathogens, like *Brachyspira hyodysenteriae* (Vangroenweghe, 2008), are re-emerging fastly. This was also observed in Sweden and Denmark, where a voluntary ban was set in the late 90's which was accompanied with a sharp increase in theurapeutic use of antibiotics (in review by Casewell *et al.,* 2003). Moreover, in the EU antimicrobial levels of copper for pigs younger than 12 w (170 mg/kg) are foreseen to be reduced in the near future, this for reasons of resistance development and accumulation in agricultural soils. Hence, there was and still is an urgent need for new ways to guarantee performance and to protect the health status of animals, especially in the more vulnerable stages of production, such as the weaning period of piglets. This target can be attained by good housing or climate conditions along with a fine selection of feedstuffs and well balanced feeds.

'Alternative' feed additives can also play a prominent role. Long before the ban on AMGP research groups came up with new substances and tested their efficacy as replacers for the AMGP. The following list of the so called 'alternative' feed additives is by no means exhaustive: enzymes, probiotics, prebiotics (fermentable carbohydrates), antibodies, spray-dried porcine plasma, cytokines, metals like Zn and Cu, acidifiers (organic acids), medium-chain fatty acids, fermented liquid feed, herbs, plants extracts and bateriophages (for reviews see: Close, 2000; Gill *et al.*, 2005; Roselli *et al.*, 2005; Thomke & Elwinger, 1998; Wenk, 2003 and many others). Not all mentioned alternatives mimic the presumed mode of action of antibiotics, but can act at different sites of the GIT and relay on different targets such as modifying the intestinal microbial balance in favour of potentially beneficial bacterial strains (competitive exclusion by probiotics and stimulating the growth of desirable bacteria by prebiotics), competitive colonization of the mucosa by adhering non-pathogens (probiotics), occupation of specific receptors on mucosal surface (prebiotics), stimulation/priming of

intestinal immune response (pro- and prebiotics, herbs and plant extracts), altering intestinal physico-chemical conditions to suppress pathogenic bacteria (acidifiers, Cu and metabolic products from fermentation of prebiotics), production of bacteriocins (probiotics), alleviation of inflammatory response (Zn, herbs and plant extracts), improving nutrient digestibility and consequently lowering nutrient availability for opportunistic bacteria (enzymes) and antioxidant activity (herbs and plant extracts). Some of the alternatives described may be part of an overall adapted strategy in pig raising or may be combined. The use of AMGP is evidenced by their consistent positive impact on animal growth, feed efficiency and disease susceptibility. The recent alternatives do not always include these benefits, but are generally aiming at healthier pigs which can better overcome the post-weaning growth check and show a more balanced gut health status. Up to date, no product has been successful in replicating the relatively consistent and robust effects on performance of the AMGP (Dibner & Buttin, 2002).

Herbs, plants extracts and essential oils (EO) have been used for food preservation, pharmaceuticals, alternative medicine and natural therapies for many years (Jones, 1996). They also have been proposed and reviewed as alternatives for the antibiotics in pig raising (Doyle, 2001; Kamel, 2001; Lis-Balchin, 2003; Rodehutscord & Kluth, 2002; Turner *et al.*, 2001; Wenk, 2003; Westendarp, 2005 and Windisch *et al.,* 2008). However, different modes of action (*e.g.* antioxidant, antimicrobial, coccidiostatic, anti-inflammatory, immuno-modulating effects and enhancement of endogenous secretions) are described depending on the product considered. A large quantity of compounds from herbal origin are well known for their antimicrobial properties (for review see Burt, 2004; Cowan, 1999; Hulin *et al.*, 1998 and Nychas & Skandamis, 2003) with the EO and their components being a very interesting and promising group. EO are the odorous components found in various plant parts. Because they evaporate when exposed to the air at ambient temperatures, they are also called volatile oils or ethereal oils. As a rule, they are immiscible with water, however, they are soluble in ether, alcohol and most organic solvents. EO in plants, form a very complex mixture which

can contain about 20-60 or more components at quite different concentrations. Components are mostly hydrophobic with a terpene, terpenoid or phenylpropene nature. EO are characterized by two or three major components at fairly high concentrations (20-80%) in addition to others present in trace amounts. Generally, these major components determine the biological properties of the EO.

In this research 5 pure EO components were selected, based on their antibacterial properties found in literature and studied for their possible application as alternative for the AMGP. The EO are the **terpenoids carvacrol and thymol and the phenylpropenes anethole, eugenol and cinnamaldehyde**. These compounds can be isolated from plant extracts; however the ones used in this research were synthetically derived ('natural identical') (see Chapter 1 for biological and synthetic synthesis and isolation). Carvacrol is antibacterial. It is added to different products, *e.g.* baked good (15.75 mg/kg), non-alcoholic beverages, chewing gum (Ultee *et al.*, 1999), as fragrance in cosmetic products and is also used in organic syntheses (Andersen, 2006). Thymol is an antifungal and antibacterial agent. It is widely used to treat the *Varroa* mite in honey bee cultures (Imdorf *et al.*, 1999). It is included in topical antiseptic and analgesic preparations in concentrations ranging from 0.1 to 1%, in personal-care products, and in mouthwashes (in conjunction with chlorhexidine) for its antiseptic action (Robbers *et al.*, 1996) and added to food products as flavouring and adjuvant (Andersen, 2006). Thymol is added as a stabilizer to several therapeutic agents, including halothane. Further, it is also used for inhalation in respiratory disorders. Its veterinary use includes the treatment of bronchitis, laryngitis, infections of the upper respiratory system, pleuritis and pneumonia with a recommended dose of 10 mg per animal for 5 consecutive days, irrespective of species (Anonymous, 1996). At one time, thymol was much used in hookworm (*Ancylostoma duodenale*) treatment (Evans, 2002). Anethole is widely used by industry to enhance the flavour of foods and alcoholic beverages, and as an odorant in perfumes (Soares *et al.*, 2007). Eugenol, as a component of clove oil, is used to relief toothache due to its anaesthetic and antiseptic properties. Further, it is widely used as a component of zinc oxide eugenol cement in dental filling and in cosmetic and food products. The

average maximum used levels in food products, such as beverages, ice cream, baked goods, gelatins and puddings, and chewing gums, range from 1.4 to 500 mg/kg. Eugenol is the starting material in the synthesis of vanillin. Cinnamaldehyde is a major flavouring agent and it is extensively used in beverages, ice creams, confectionery, baked foods, chewing gums, condiments and meat preparations at concentrations from 8 mg/kg in ice cream to 700 mg/kg in boiled sweets. Its main application is as fragrance material in cosmetics, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. It is also used as animal repellent, insect attractant and antifungal agent. Cinnamaldehyde is extensively studied as a model molecule for the selective hydrogenation of *α,β*-unsaturated aldehydes. More recently, all these compounds have gained interest as antimicrobial inclusion agent in edible coatings (*e.g*. Ben Arfa *et al.*, 2007; Du *et al.*, 2008 and Rojas-Grau *et al.*, 2007) and their effects towards various invertebrates (nematodes, snails, insects …) and weed seedlings are intensively investigated.

Objectives and outline of the thesis

The **specific objectives** of the present study are:

[1] to characterise the antimicrobial activities of EO against piglet intestinal bacteria;

[2] to study the stability and kinetics of EO in the GIT of piglets and

[3] to study the effect of EO on the functionality of the gut in piglets.

Improved insight into the biological effects and kinetics of EO should allow to better evaluate the potential of EO as replacers of the AMGP in pig nutrition.

Concerning the experimental work, effects were mainly quantified related to the weaning period of pigs, because in current pig production systems, the weaning period is one of the main critical points for growth and survival of the animal.

In **Chapter 1**, a literature review is presented. It comprises relevant data of carvacrol, thymol, anethole, eugenol and cinnamaldehyde, with special emphasis on the antimicrobial activities and the reported effects on pig gut bacteria and physiology and pig performance. Data on effects in birds (poultry) are not included, since a lot of dissimilarities in digestive anatomy and function between mammals and birds exist.

Chapter 2 describes experimental work dealing with the antimicrobial activity against the main culturable components of the pig gut bacteria. *In vitro* incubations, simulating the fermentation in different sections of the GIT, to study the effects of added EO on the bacteria were carried out. Firstly, a screening study was performed with carvacrol, thymol, eucalyptol, terpinen-4-ol, *E*-anethole, eugenol and *E*-cinnamaldehyde. Dose-response relations were established and combinations were tested in a second study with the most promising EO.

Data on the stability and kinetics of carvacrol, thymol, eugenol and *E*-cinnamaldehyde in the GIT of pigs were determined in a subsequent study (**Chapter 3**). Samples of *in vitro* incubations were analysed for their EO content, and the recovery was calculated as a measure for stability/degradation. A single dose of each of these EO mixed with feed was given to piglets. Following euthanasia at different times post-prandial, digesta and body fluids were sampled to obtain kinetic data *in vivo*.

A choice-feeding trial was done in which weaned piglets could freely choose between a control and an experimental diet. The objective was to determine whether a diet supplemented with thymol would be preferred or not, and this in relation to the dose applied. Further, flavours were added to mask thymol and tested. This work is reported in **Chapter 4A**.

Two *in vivo* trials with carvacrol and thymol for newly-weaned piglets are described in **Chapter 4B**. Doses of 500 and 2000 mg/kg feed and different formulations were tested. Effects on gut bacteria and functionality were gathered by microbiological and biochemical analysis of digesta and body fluids and histological examination of the gut tissue.

Chapter 5 presents a detailed study of the effects of thymol, *E*-anethole and *E*-cinnamaldehyde on active epithelial absorption of D-glucose and L-alanine and stimulated chloride secretion. For that purpose, the Ussing chamber technique was used.

Finally, a general discussion (**Chapter 6**) relates all data. Data are viewed in the perspective of these EO as alternatives for the AMGP and future prospects are discussed.

CHAPTER 1

ESSENTIAL OILS AS FEED ADDITIVES IN PIG NUTRITION

CHAPTER 1

ESSENTIAL OILS AS FEED ADDITIVES IN PIG NUTRITION

1. FATE OF SELECTED ESSENTIAL OILS IN PLANTS AND PROPERTIES

1.1. Biosynthesis

EO are the odorous, volatile products of an aromatic **plant's secondary metabolism**, normally formed in special cells or groups of cells, found in many leaves and stems. The production of plant secondary constituents is dependent on the fundamental metabolic cycles of the living plant tissues. A number of recent reports suggest that secondary metabolites, rather than constituting waste products of metabolism, are biosynthesized to aid to the producer's survival (Evans, 2002). Recently, considerable attention has been directed to the possible ecological implications of secondary metabolites not only in relation to plant-plant interaction but also concerning the interrelationship of plants and animals.

Carvacrol and thymol (Table 1) are classified as **monoterpenoids** and their biosynthesis in plants follows the general scheme of terpenoid synthesis (Robbers *et al.*, 1996). Terpenoids are composed of isoprene units; monoterpenoids having two units. The **isoprene units** arise from acetate via **mevalonic acid** and are branched-chain, five carbon units containing two unsaturated bonds (Fig 1.). Isopentenyl-5-pyrophosphate (IPP) is termed active isoprene. Geranyl pyrophosphate serves as the substrate for the various monoterpenoid synthases (mainly cyclases) and represents the key precursor

of monoterpenoid biosynthesis. Geranyl pyrophosphate itself cannot cyclize directly to form a six-membered ring because of the *trans*-geometry of the double bond at C-2. However, the monoterpenoid synthases isomerize it to a bound intermediate with the *cis*-geometry, which is then able to cyclize. After initial cyclizations, these compounds can undergo a variety of subsequent enzymatic transformations including oxidations, reductions … The synthesis of carvacrol and thymol follows the aromatization of their precursor *γ*-terpene to *p*-cymene and finally the hydroxylation of the latter. Carvacrol and thymol, although aromatic in structure, are thus terpenoid in origin.

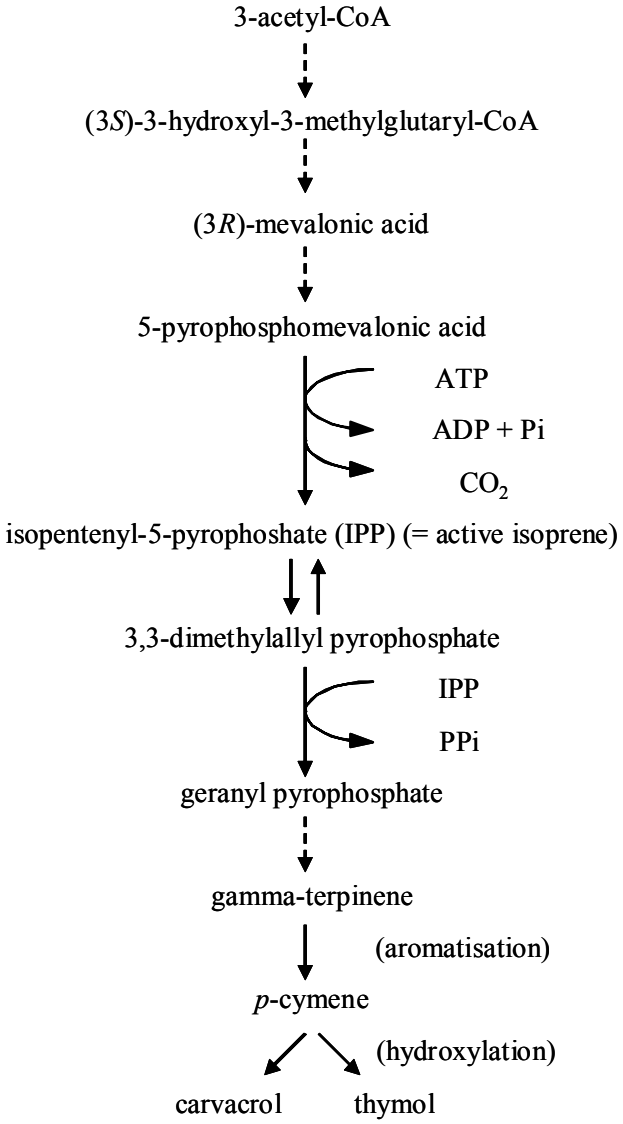


Fig. 1. Biosynthesis in plants of carvacrol and thymol follows the general scheme of terpenoid synthesis (adapted from Robbers *et al.,* 1996).

Anethole, eugenol and cinnamaldehyde (Table 1) are **phenylpropanoids** formed in plants via the **shikimic acid-phenylpropanoid route** (Fig. 2) (Robbers *et al.*, 1996). Phenylpropanoids contain the C_6 phenyl ring with an attached C_3 propane side chain. Shikimic acid, through a series of phosphorylated intermediates, yields chorismic acid, which is an important branch-point intermediate. One branch leads to prephenic acid that can be aromatized in two ways. The first proceeds by dehydration and simultaneous decarboxylation to yield phenylpyruvic acid, the direct precursor of L-phenylalanine. Cinnamic acid, is then formed by direct enzymatic deamination of phenylalanine. The acid may then undergo a number of elaboration reactions to generate many of the phenylpropanoids; for example cinnamic acid is reduced to the corresponding aldehyde, cinnamaldehyde. The second occurs by dehydrogenation and decarboxylation to yield *p*-hydroxyphenylpyruvic acid, the precursor of L-tyrosine. *P*-hydroxycinnamic acid (*p*-coumaric acid) can originate from tyrosine or by hydroxylation of cinnamic acid at the para position. Anethole and eugenol are derived from *p*-hydroxycinnamic acid. The principal precursors for phenylpropanoids are therefore cinnamic acid and *p*-hydroxycinnamic acid, which arise in turn from the aromatic amino acids L-phenylalanine and L-tyrosine. For anethole and cinnamaldehyde two enantiomeric forms exist, *E (=trans)* and Z (*=cis)*. However, in nature ***E*** is the predominant isomer (>99%) for both compounds (Aromatic Consortium, 2005).

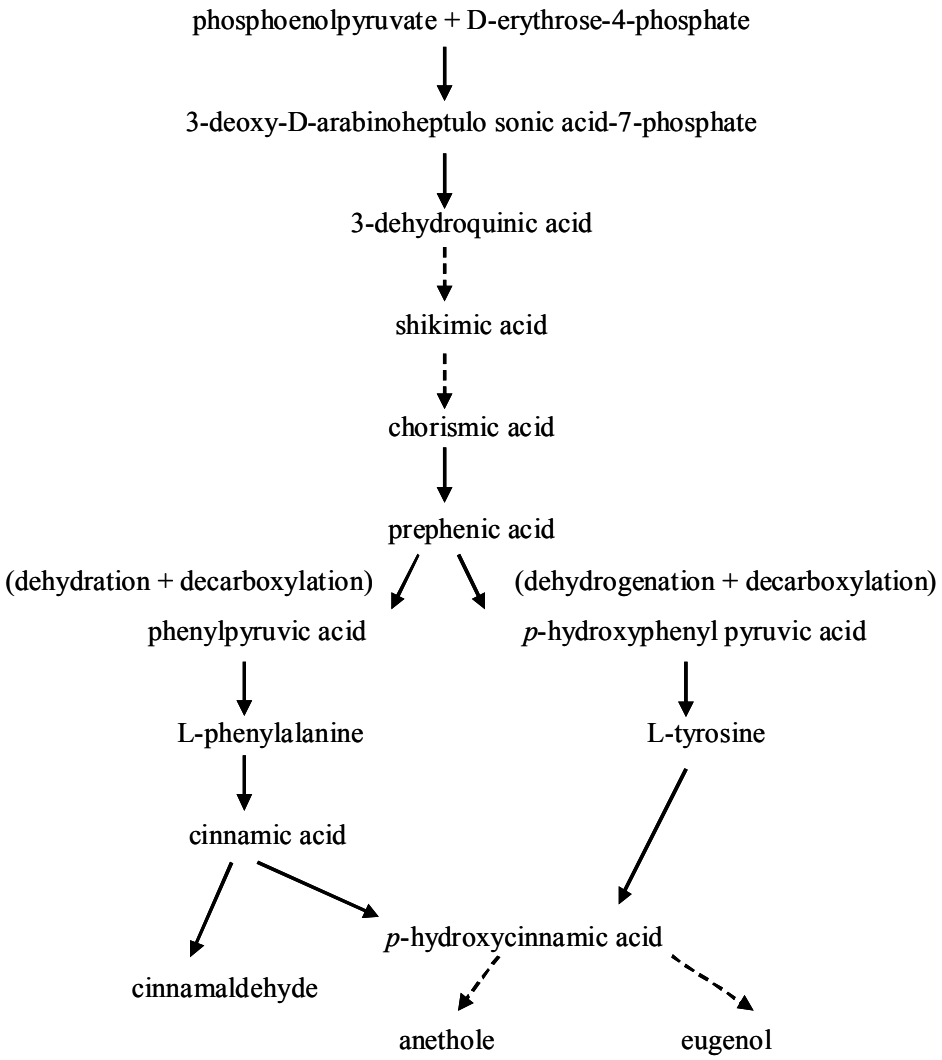


Fig. 2. Biosynthesis in plants of anethole, eugenol and cinnamaldehyde via the shikimic acid-phenylpropanoid route (adapted from Robbers *et al.,* 1996).

1.2. Presence in plants and synthetic analogues

The production of secondary constituents of plants are influenced by three principal factors: genetic composition (chemotypes), ontogeny (stage of development) and environment (soil, climate, associated flora and methods of cultivation) (Robbers *et al.*, 1996). For many EO containing plant species, distinct chemotypes are described. Chemotypes are defined as chemically distinct populations within a species and have similar phenotypes but different genotypes and as such are

identical in external appearance but differ in their constituents of the isolated plant EO (Evans, 2002). Genetic effects induce both quantitative and qualitative changes, but those caused by environmental influences are primarily quantitative. Stage of development depends on harvesting time. To conclude, it means that the composition of the plant EO is variable and this should be taken into account, when exploiting an EO. In practice, EO are commonly extracted from plant material by pressure, extraction, steam distillation or supercritical carbon dioxide and compose of an often quite complex mixture of mostly hydrophobic chemicals with a terpene, terpenoid, phenylpropene or phenylpropanoid nature. Only a few posses a single component in a high percentage (*e.g.* clove oil contains no less than 85% of phenolic substances, chiefly eugenol). However, it is not uncommon for a EO to contain over 200 components.

Carvacrol is present in the EO obtained from several species of the genus *Origanum*, *e.g. O. vulgare, O. intercedens, O. ornites and O. marjoram.* This genus belongs to the Lamiaceae family. The flowering plant is harvested and the carvacrol content is different for leaves and flowering tops. Carvacrol is the major component of oregano oil, principally 50 to 80%. Oregano oil contains also thymol and the precursors *γ*-terpinene and *p*-cymene to varying concentrations (*e.g.* Sivropoulou *et al.*, 1996). Carvacrol is present in thyme oil (9-60%), as well in many other plant species; *e.g. Coridothymus capitatus* (70%), *Satureja montana* (22-40%) (Peňalver *et al.*, 2005). Carvacrol is synthetically derived from *p*-cymene by sulfonation followed by alkali fusion (Andersen, 2006).

Thymol is obtained from thyme oil (*Thymus vulgaris* L. and *Thymus zygis*; both are included in the *European Pharmacopoeia - EP* and *British Pharmacopoeia - BP*), from horsemint oil (*Monarda punctata* L.), from Oswego tea oil (*Monarda didyma* L.), from ajowan oil (*Carum copticum* B. et B.) and many others. A number of chemical varieties, *e.g.* 'thymol' and 'carvacrol' types, are known for *Thymus*. The genus *Thymus* belongs to the family Lamiaceae. Thyme oil is obtained from the leaves and flowering tops of the plant and commonly contains 20-55% thymol, accompanied by lower

amounts of carvacrol, *γ*-terpinene and *p*-cymene. The thyme oil may be treated in two ways to isolate thymol crystals: [1] it may be subjected to freezing temperatures causing the thymol to crystallize or [2] it may be treated with sodium hydroxide solution, the aqueous solution of sodium thymol being separated and decomposed with acid, thus liberating the thymol, which is subsequently purified. Thymol may be prepared synthetically from *m*-cresol and isopropyl chloride by the Fiedel-Crafts method at -10°C or more efficiently from *m*-cresol and propene at elevated temperatures (Andersen, 2006).

It is increasingly evident that both carvacrol and thymol also occur in plants in the glycoside form. Both were found in *T. vulgaris* L. as glucosides and galactosides (Evans, 2002) and carvacrol and thymol glycosides were also detected in *S. montana* (Mastelic & Jerkovic, 2003) and *O. vulgare* (Milos *et al.*, 2000). These glycosides are translocated from leaves to flowers as aroma precursors and serve as a means of translocation/storage within the plant. Glycosides are water soluble, non-volatile and flavourless and offer therefore some advantages compared to the parent compound. A reliable differentiation of natural from synthetic carvacrol and thymol is feasible by multi-element GC-IRMS (Gas Chromatography – Isotope Ratio Mass Spectrometry) on $^{2}H/^{1}H$, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios relative to international standards (Nhu-Trang *et al.*, 2006; Greule *et al.*, 2008). Determination of the isotope ratios (only $^{2}H/^{1}H$ ratios are practicable) of the actual compound allows a discrimination between natural (isolated from plants) or synthetic (with synthetic compounds as starting material) origin, thus assessing its authenticity.

Pimpinella anisum L. (aniseed, Apiaceae) and *Illicium verum* Hook (star-anise, Illiciaceae) are important sources of anethole (both included in *EP* and *BP*). Aniseed oil is obtained from the dried, ripe fruits of the plant and yields commonly 2-5% of EO. The *Pimpinella* oil is said to have a slightly superior flavour than the *Illicium* oil, but most of the aniseed oil used is that obtained from the star-anise. Anethole is the principal constituent in the oil (80-90%). The oil is sensitive to atmospheric

oxidation and both anisic aldehyde and anisic acid are normally present. This change is said to diminish the tendency of the oil to solidify, which it normally does on cooling to about 15°C. Oil of anise is used as a flavouring agent and carminative (a herb or preparation that is able to combat flatulence). Anethole is also the major compound of fennel oil (*Foeniculum vulgare*), about 60%. All these oils are components of popular Mediterranean beverages such as anisette, ouzo and raki. Besides isolation from the mentioned plant EO, anethole can be obtained by isolation from crude sulphate turpentine or chemically derived by treatment of anisole with propionic acid derivates or propionaldehyde (Terpene Consortium, 2002).

Clove is the dried flower bud of *Eugenia caryophyllata* B. et H. (*Syzygium aromaticum*), family Myrtaceae, and is the main source of natural eugenol. Clove oil is the EO distilled with steam from the dried flower buds (yield 14-21%, >80% eugenol) (official in *EP* and *BP*). Oils with a particularly high content of eugenol are used in the commercial production of vanillin. Eugenol is usually prepared from clove oil by shaking with a 10% solution of sodium hydroxide to form sodium eugenolate (Robbers *et al.*, 1996). The mixture is washed with ether, and the sodium eugenolate is then decomposed with sulphuric acid. Eugenol is separated by steam distillation. It has a strong aromatic odour of clove and a pungent spicy taste. Cinnamon leaf oil contains 70-95% of eugenol as well. Eugenol occurs as glucoside in *Melissa officinalis* (Evans, 2002). Eugenol can be synthetically derived from benzoic acid.

Cinnamaldehyde is present in different species of the genus *Cinnamomum* (Lauraceae). Ceylon cinnamom refers to *Cinnamomun zeylanicum* B. (*Cinnamomun verum*), the dried inner bark of shoots of coppiced trees is used for the production of the Ceylon cinnamon oil (yield 0.5-1%). The principal constituent of the oil is cinnamaldehyde (60-95%), the remainder consists of terpenes such as limonene, *p*-cymene, (-)-linalool and *β*-caryophyllene and other compounds such as eugenol. Cinnamon oil is used as a flavouring agent; it is also a carminative and pungent aromatic. It has

antiseptic properties. Cassia oil is derived from *Cinnamomun cassia* N. Cassia yields 1-2% of EO which, when pure, contains no eugenol but rarely less than 85% of cinnamaldehyde. Cinnamaldehyde can be isolated by sequential fractionation with various solvents and silica gel column chromatography. On a commercial scale, cinnamaldehyde is prepared almost exclusively from the alkaline condensation of benzaldehyde and acetaldehyde (Aromatic Consortium, 2005).

1.3. Physico-chemical properties and stability

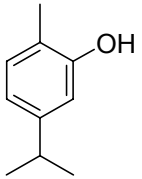
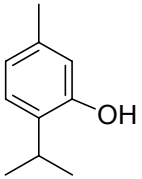
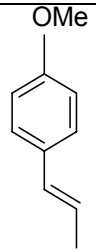
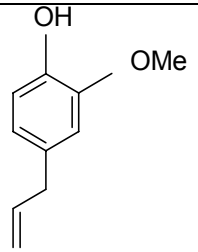
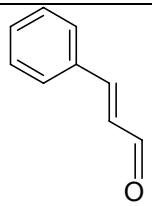
An overview of the physico-chemical properties of the pure forms of the EO is shown in Table 1. All compounds contain a benzene unit which provides their **aromatic nature**. In general, they are **volatile** and are prone to volatilization. Carvacrol and thymol are isomers bearing the hydroxyl group at a different position. Both are phenolic compounds with a moderate solubility in water and a pKa value of 10.4. Si *et al.* (2006b) found that pre-incubation of carvacrol and thymol in a pH 2.0 medium at 37°C for 4h did not alter the efficacy of antimicrobial activity. Thymol was susceptible to ozonation (Nakada *et al.*, 2007). Anethole and eugenol have both a methoxy moiety. Anethole has a much lower water solubility than the other EO used in this research. When water is added to anise containing drinks (anisette, ouzo …), a cloudy white suspension results which is attributable to a decrease in solubility of this phenylpropanoid as it is much more soluble in ethanol than in water. Anethole should be stored air- and light-protected to avoid oxidation. The calculated half-life value for *E*-anethole has been reported to be as short as 2.0 h; it contains a reactive allylic hydrogen capable of ready reaction with hydroxyl and peroxy radicals (Terpene Consortium, 2002). Oxidation of anethole and eugenol was effected under photochemical conditions using hydrogen peroxide to obtain either epoxy or hydro-epoxy derivates (Elgendy & Khayyat, 2008). Eugenol has a phenol moiety as well and typically its density exceeds that of water. Eugenol was resistant to degradation when exposed to temperatures up to 200°C (Friedman *et al.*, 2000) and its antimicrobial properties were not affected by pre-incubation at pH 2.0 (Si *et al.,* 2006b).

Cinnamaldehyde contains an oxidizable *α,β*-unsaturated aldehyde function. The calculated photodegradation half life is around 3 h, the *α,β*-unsaturated aldehyde function being susceptible to attack by hydroxyl radical species in the gas phase (Aromatic Consortium, 2005). No hydrolysis is possible for cinnamaldehyde, so it is expected to be stable in an aqueous medium, although it may be slowly oxidized to cinnamic acid (Aromatic Consortium, 2005). Yuan *et al.* (1992) did not find any oxidation of cinnamaldehyde to cinnamic acid in aqueous solutions. However, in commercial applications (as flavouring agent) antioxidants are added as stabilizers (Aromatic Consortium, 2005). Temperature-dependent and non-enzymatic oxidation and further decomposition to benzaldehyde and glyoxal has been reported by Friedman *et al.* (2000) and Gholivand & Ahmadi (2008), occurring at temperatures above 60-70°C. Benzaldehyde itself has the bitter almond odor. Interestingly, Friedman *et al.* (2000) found also that cinnamaldehyde was not heat degraded when present in cinnamon oil or when mixed with eugenol. Basketter (2000) reviewed the quenching effect of eugenol towards the skin sensitizing activity of cinnamaldehyde in human. Due to its electrophilic groups, cinnamaldehyde can react directly with proteins via two different mechanisms: Schiff base intermediates to lysine residues and Michael addition to sulfhydryls (Elahi *et al.*, 2004). Pre-incubation of cinnamaldehyde in a pH 2.0 medium at 37°C for 4h did not alter the efficacy of antimicrobial activity (Si *et al.*, 2006b).

Systematic research on the **stability** of these compounds **during feed processing (for example pelletizing) and feed storage** conditions has rarely been done. However, considering the available data above mentioned, adequate research might be necessary; especially for *E*-anethole and *E*-cinnamaldehyde. Wald (2002) found that 20 to 24% of carvacrol and thymol within OEO (oregano essential oil) was lost by pelletising the feed at 70 and 90°C. It was shown that losses of carvacrol (18%) and eugenol (18-27%) were not due to the pelletising step (81 to 86°C) but caused by the preceding steam treatment (48 to 55°C) (Bauerman, 2006). During storage of pelleted feed

containing app. 44.4 mg/kg carvacrol for 7 weeks, there was no loss of this compound, in contrast to *γ*-terpene and *p*-cymene (Westendarp *et al.,* 2006).

Table 1. Physico-chemical properties of selected essential oils

Compound (IUPAC; CAS No.)	Carvacrol (2-methyl-5-propan-2-ylphenol; 499-75-2)	Thymol (5-methyl-2-isopropylphenol; 89-83-8)	Anethole (1-methoxy-4-(1-propenyl)benzene; 104-46-1)	Eugenol (4-allyl-2-methoxyphenol; 97-53-0)	Cinnamaldehyde (3-phenylprop-2-enal; 104-55-2)
Chemical structure					
Formula	$C_{10}H_{14}O$	$C_{10}H_{14}O$	$C_{10}H_{12}O$	$C_{10}H_{12}O_2$	C_9H_8O
Molecular weight (D)	150.2	150.2	148.2	164.2	132.2
Density (g/cm^3)	0.976	0.969	0.988	1.067	1.050
Melting point (°C)	0-2	49-52	23	-12 - -10	-7.5
Boiling point (°C)	234-238	232-233	234-237	253	246-251
Vapour pressure at 20°C (Pa)	35	250 (50°C) - 133 (64°C)	4.9	133 (78°C)	3.85
Sol. in water (g/L)	0.83-1.10	0.85-1.01 to 1.4 (40°C)	0.11	0.80-2.41	1.42-1.45
Sol. in ethanol (g/L)	good	1000	good	500 (in 70%), good	150 (in 60%), good
Octanol/water partition coefficient (log K_{ow})	3.38-3.64	3.30	-	2.99	1.9
pKa value	10.4	10.4	-	-	-
Physical appearance at room temperature	colourless to pale yellow liquid	white crystalline powder or large colourless crystals	colourless liquid	colourless or pale yellow, thin liquid	clear yellowish liquid

Source: Andersen, 2006; Anonymous, 2000; Aromatic Consortium, 2005; Cocchiara *et al.,* 2005; Griffin *et al.,* 1999; Helander *et al.*, 1998; Knobloch *et al.*, 1989; Lee *et al.*, 2004; Terpene Consortium, 2002; Ultee *et al.*, 2002 and MSDS of product nr. 282197, T0501, 117870, E51791 and 96320 (Sigma-Aldrich, Bornem, Belgium)

2. ANTIMICROBIAL ACTIVITY OF SELECTED ESSENTIAL OILS

2.1. Mode of action

The mode of action of the phenolics carvacrol and thymol involves several target sites, mainly acting on the bacterial cell membranes. Important to note is that the cytoplasmic membrane of bacteria has two principal functions: [1] barrier function and energy transduction, which allow the membrane to form ion gradients that can be used to drive various processes, and [2] formation of a matrix for membrane-embedded proteins (Sikkema *et al.*, 1995). The first effort was done by Juven *et al.* (1994) who examined the working of thymol against *Salmonella* Typhimurium and *Staphylococcus aureus*. These authors hypothesised that thymol binds to membrane proteins hydrophobically by means of hydrogen bonding, thereby changing the permeability characteristics of the membrane. Further, they stated that carvacrol and thymol sensitize the membranes and when saturation of the site(s) of action occurs, there is a gross damage and sudden collapse of the integrity of the bacterial cytoplasmic membrane and leakage of vital intracellular constituents. Secondly, as a result of their lipophilic structure, these components will partition easily into the bacterial membranes (between the fatty acid chains) and make the membranes to expand and become more fluid. These two actions lead to membrane expansion, fluidization, destabilization and permeabilization, followed by release of membrane-associated and cell material, which has been reported in several studies (Cristani *et al.,* 2007; Gill & Holley, 2006a; Helander *et al.*, 1998; Rhayour *et al.*, 2003; Trombetta *et al.*, 2005; Ultee *et al.*, 2002; Veldhuizen *et al.*, 2006; Walsh *et al.*, 2003 and Xu *et al.,* 2008). Knobloch *et al.* (1989) showed that carvacrol and thymol had an inhibitory effect on enzymatic reactions catalyzed by cell-free bacterial membranes. The studies of Ultee *et al.* (1999) and Ultee *et al.* (2002) on the action of carvacrol on *Bacillus cereus* led to the hypothesis of the 'proton exchanger model' (Fig. 3), whereby carvacrol acts as a trans-membrane carrier of monovalent cations by exchanging its hydroxyl proton for another ion such as a potassium ion (thus acting like an ionophore) leading to

dissipation of the pH gradient and electrical potential over the membrane, loss of the proton motive force and depletion of intracellular ATP pools, which is the onset to impairment of essential cell processes. Loss of potassium is problematic as well, since it plays a role in the activation of cytoplasmatic enzymes, in maintaining osmotic pressure and in the regulation of cytoplasmic pH. In most cases, bacteria can counterbalance these effects by using ionic pumps and cell death does not always occur, but large amounts of energy are diverted to this function and bacterial growth is slowed down. Furthermore, a 2 h treatment of several bacteria with bactericidal levels of carvacrol and thymol induced a decrease in the proportion of unsaturated fatty acids (UFA) in the membrane (Di Pasqua *et al.*, 2007). We conclude that the **primary mechanism of action for carvacrol and thymol** is therefore clearly related to its **effect on the cytoplasmic membranes and energy generation**.

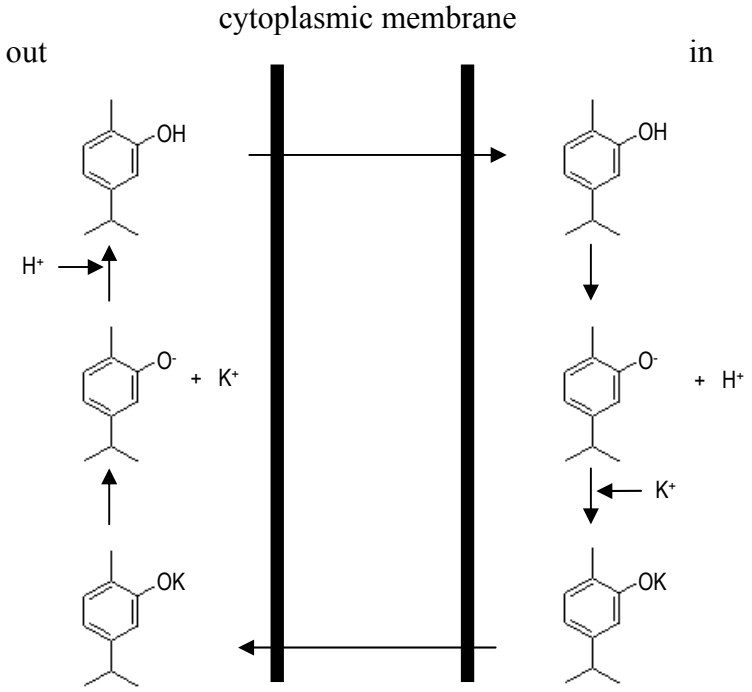


Fig. 3. Schematic overview of the hypothesized activity of carvacrol. Undissociated carvacrol diffuses through the cytoplasmic membrane towards the cytoplasm and dissociates, thereby releasing its proton to the cytoplasm. It then returns undissociated by carrying a potassium ion or other cation from the cytoplasm to the external environment (Ultee *et al.,* 2002).

Nevertheless, secondary effects of these phenolics, necessary for the bactericidal activity cannot be discounted. Gill & Holley (2006b) found that significant inhibition of ATPase activity of *Escherichia coli* and *Listeria monocytogenes* by carvacrol occurs at concentrations within the same range required for membrane disruption (> 750 mg/L), and suggested that this is a secondary cause of cell death. Helander *et al.* (1998) and Trombetta *et al.* (2005) stated that carvacrol and thymol might cross the cell membranes, penetrating the interior of the cell and interacting with intracellular sites probably critical for antibacterial activity. It has been demonstrated that highly lipophilic and low molecular weight compounds penetrate relatively easily through the outer membrane of several G- bacteria (Sikkema *et al.*, 1995) and moreover carvacrol and thymol were also able to disintegrate the outer membrane of two G- bacteria (*S.* Typhimurium and *E. coli* O157:H7) thereby releasing lipopolysaccharides (Helander *et al.*, 1998). A sharp decrease (80%) in *B. cereus* diarrheal toxin production was observed in the presence of 0.06 mg/mL carvacrol (Ultee & Smid, 2001). Overnight incubation with carvacrol induced production of heat shock protein 60, but not heat shock protein 70, and inhibited the synthesis of flagellin in *E. coli* rendering the bacterium non-motile and less infective (Burt *et al.*, 2007b). To study further the mode of action of thymol, Shapira & Mimran (2007) constructed *E. coli* mutans exhibiting altered response to thymol. Essentially, they demonstrated that the antimicrobial activity of this compound cannot be attributed solely to depolarization of the membrane (membrane effect).

For eugenol which has a phenolic functional group, membrane effects have been reported causing potassium leakage from *E. coli* (500 mg/L) and *S. aureus* (1000 mg/L) (Walsh *et al.*, 2003) and depletion of intracellular ATP of *E. coli* (Gill & Holley, 2006a) and are considered the primary mechanism of action. Gill & Holley (2004) suggested that depletion of intracellular ATP by eugenol could be primarily due to ATPase inhibition, more likely than by membrane effects dissipating the proton motive force. **Eugenol and cinnamaldehyde** induced a decrease of UFA in the bacterial membrane after a 2 h treatment (Di Pasqua *et al.*, 2007). Nevertheless, **secondary effects are here**

more important and diverse. Despite the vast data about the antimicrobial activity of cinnamaldehyde, the mode of action of this substance is much less understood and current knowledge points out that a range of independent and likely species-dependent targets sites are involved. Helander *et al.* (1998) found that cinnamaldehyde did not exhibit outer membrane disintegrating activity nor depletion of intracellular ATP, but suggested that cinnamaldehyde gain access to the periplasm and deeper parts of the cell through porin proteins. Becerril *et al.* (2007) retrieved this compound in dead *E. coli* cells, so the compound must penetrate the bacteria before cell disruption takes place. Electron microscopic observations revealed that the bacterial cells treated with the cinnamaldehyde also suffered from severe damages in their surface structure (Kim *et al.*, 2004), but the membrane permeabilization observed by Gill & Holley (2006a) was clearly not as definitive as for eugenol and carvacrol. For compounds with an aldehyde group conjugated to a carbon to carbon double bond (*α,β*-unsaturated aldehyde) resulting in a highly electronegative arrangement like cinnamaldehyde, it is known that they may interfere in biological processes involving electron transfer and react with vital nitrogen components, *e.g.* functional groups on proteins and nucleic acids and therefore inhibit the growth of micro-organisms (Lis-Balchin, 2003) or form Schiff's bases with membrane proteins and so prevent cell wall biosynthesis (Friedman, 1996). Treatment of cinnamaldehyde to *B. cereus* exponential phase cells resulted in no significant protein leakage but strong inhibition of cell separation (Kwon *et al.*, 2003). Gill & Holley (2004) hypothesized that the mechanisms of the observed rapid inhibition of energy generation of cinnamaldehyde against *L. monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei* at bactericidal concentrations could be due to the inhibition of glucose uptake or utilization and effects on membrane permeability. Gill & Holley (2006b) found that significant inhibition of ATPase activity of *E. coli* and *L. monocytogenes* by eugenol and cinnamaldehyde occurs at concentrations within the same range required for membrane disruption (> 750 mg/L) and suggested that this is rather a secondary cause of cell death. Sub-lethal concentrations of eugenol inhibited the production of amylase and proteases by *B. cereus*. Cell wall deterioration and a high degree of cell lysis were also

noted (Thoroski *et al.*, 1989). Cinnamaldehyde and eugenol were found to be very effective inhibitors of histidine decarboxylase activity of *Enterococcus aerogenes* at sublethal levels (Wendakoon & Sakaguchi, 1995). The hydroxyl group of eugenol and the carbonyl group of cinnamaldehyde are thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes*. Finally, cinnamaldehyde binds to the FtsZ protein and inhibited subsequently cell division of *B. cereus* (Domadia *et al.*, 2007).

2.2. Evidence for antimicrobial activity and structure relationship

Numerous studies have been performed to investigate the antimicrobial activities of plant EO against many different types of microbes, mainly foodborne pathogens and bacteria present in the oral cavity. Test methods used, include standardised broth dilution or disc and agar well diffusion assays. However, problems associated with the assessment of the antimicrobial activity by these diffusion methods are that the method in itself is highly dependent on water solubility and the ability of test components to diffuse through agar (Southwell *et al.*, 1993) and on the effect on bacteria of oil vapours that may be released. As outlined above, EO are moderately to highly lipophilic, so this characteristic will influence results obtained in these tests. Turbidimetry (measurements of optical density) used in broth dilution assays detects only the upper part of growth curves (> 6 $\log_{10}$ CFU/mL) (CFU, colony forming units), is influenced by the size of the bacterial cells, and requires calibration in order to correlate the results with viable counts obtained on agar media (plate counting) (Nychas & Skandamis, 2003). Therefore, **results reported can differ a lot and are difficult to compare directly,** also because different measures of bactericidal activity, broth media, redox potentials, NaCl content in the media, temperatures, pH, a_w values, bacterial strains, enumeration techniques and sources of essential oils are used. Many of these factors can greatly affect the antibacterial activity of a compound as illustrated by Santiesteban-Lopez *et al.* (2007).

Table 2 lists data on Minimal Inhibition Concentration (MIC) and Bactericidal Activity 50% (BA50), depicted from numerous studies, but by no means complete. The table displays a high number of different species tested. Data of distinct species strains/serovars are taken together since Friedman *et al.* (2002) showed that the activities of a variety of compounds appeared to be relatively similar for various strains represented within a species. Nevertheless, in other studies strain/serovar difference in susceptibility were great, *e.g.* Si *et al.* (2006b). From Table 2, it is hard to make firm conclusions, because the range of antimicrobial activity listed for each bacterium is in some cases very wide (for reasons specified above) making a meaningful comparison impossible. Individual reports were studied in detail in an attempt to answer to the following questions: [1] can the EO be ranked in order of antimicrobial activity? and [2] are G- bacteria less susceptible than G+ bacteria? Bypassing the outer membrane is a prerequisite for any solute to exert bactericidal activity towards G- bacteria. The outer membrane represents an additional barrier to lipophilic compounds. Therefore, it is generally speculated that G- bacteria will be less susceptible than G+ bacteria (as is illustrated for a high number of plant EO by Chao *et al.* (2000) and Smith-Palmer *et al.* (1998)). However, from the studies presented in Table 2 no decisive difference in susceptibility between G- and G+ bacteria was seen. Ranking of the EO varied according to the bacterium considered. Generally, it can be stated that:

[1] **many pathogenic bateria (*e.g. Clostridium perfringens, E. coli, S. enterica, S. aureus*), including antibiotic resistant strains, were more or less susceptible to several EO indicating their huge potential in the control of pathogen proliferation;**

[2] *Pseudomonas aeruginosa* is the most resistant bacterium which is due to an active efflux mechanism and the barrier function of the outer membrane (Cox & Markham, 2007)**;**

[3] cinnamaldehyde shows a more selective antimicrobial spectrum compared to the other compounds, and

[4] anethole has weaker antimicrobial activities than the other EO.

Carvacrol and thymol exhibit similar activities against all of the species tested. Typically, both compounds do not show a progressive increase in antibacterial effect with increased concentration, but rather a sudden significant reduction in viable counts or bacterial metabolic activity once a certain (critical) concentration is applied (Juven *et al.*, 1994; Macheboeuf *et al.*, 2008). Sub-MIC levels increased the lag phase as was observed for thymol (Walsh *et al.*, 2003 and Falcone *et al.*, 2007). Both phenols are amongst the most powerful monoterpenes and this may be due to the acidity of their OH-group along with their lipophilic character (Knobloch *et al.*, 1989). Ultee *et al.* (2002) argued that the OH-group and the presence of a system of delocalized electrons (benzene ring) are important for the antimicrobial activity of carvacrol, evidenced by the fact that structure analogues lacking these features have no or minor antimicrobial activities, which was confirmed by the study of Ben Arfa *et al.* (2007). However, Veldhuizen *et al.* (2006) disagreed and stated that the OH-group is not essential for activity but does have special features that add to the antimicrobial mode of action of carvacrol. Cluster analysis based on the antimicrobial activities against 4 different species grouped carvacrol and thymol in a group of non-selective terpenoids whose main discriminating molecular properties were a higher water solubility and H-bonding capacity compared to a high number of other terpenoids (Griffin *et al.*, 1999). In a disk diffusion assay both components showed clear inhibition zones towards *E. coli, S.* Typhimurium, *Rhizobium leguminosarum, S. aureus* and *Bacillus subtilis*, but were not effective against *Pseudomonas aeruginosa* (Sivropoulou *et al.*, 1996), this is in agreement with data from Table 2. The precursors *γ*-terpinene and *p*-cymene did not reduce the growth of these bacteria as was shown by Knobloch *et al.* (1989) and Juven *et al.* (1994). Interestingly, Nostro *et al.* (2004) found no difference in susceptibility to carvacrol and thymol between methicillin-susceptible and methicillin-resistant staphylococci.

Si *et al.* (2006b) found that *Lactobacillus plantarum, Lactobacillus acidophilus* and *Bifodobacterium* spp. were less sensitive to carvacrol (200-300 mg/L), thymol (200-300 mg/L) and eugenol (300-400 mg/L) than the pathogenic bacteria *E. coli* and *S.* Typhimurium. This selective activity was most

clear for eugenol, although its bactericidal effect against the G- bacteria was weaker compared to carvacrol and thymol. Thymol showed moderate inhibition against the G+ bacteria and its activity was highest under aerobic conditions. Eugenol-isoeugenol and anethole-estragole are isomers, each of which differ only with regard to the position of the double bond in the aliphatic side chain. Eugenol and estragole have the double bond at the 2-position and isoeugenol and anethole at the 1-position. Friedman *et al.* (2002) showed that eugenol was more active than isoeugenol and that anethole was less active than estragole. Apparently, these differences can be related with the position of the double bond. Methyl- and acetyl-eugenol were less effective than eugenol indicating the importance of a free OH-group (Knobloch *et al.*, 1989). Obligate anaerobes were generally more sensitive to thymol and eugenol than facultative anaerobes (Shapiro *et al.,* 1994).

Lee & Ahn (1998) showed that cinnamaldehyde had a pronounced inhibitory activity in human *C. perfringens* and *Bacillus fragilis*, while it had no or a moderate effect against *Bifodobacterium longum* and *L. acidophilus*. Eugenol had in the same disk diffusion assay no effect against these bacteria. In humans, clostridia are associated with a variety of diseases, while bifodobacteria are considered beneficial. Therefore, this **selective inhibition by cinnamaldehyde may have a beneficial role in balancing the intestinal microbiota**. In an impregnated paper disk method, Lee *et al.* (2001) compared the antibacterial activity of several cinnamic acid derivates against human intestinal bacteria. Against *E. coli,* the order was 4-hydroxy-3-methoxy cinnamic acid > cinnamaldehyde > 3,4-dihydroxycinnamic acid > (cinnamic acid and others; not active); showing the importance of the methoxyl group. These three compounds were equally active against *C. perfringens*, but only cinnamaldehyde was (moderately to strong) active against the G+ bacteria *B. bifidum, B. acidophilus* and *L. acidophilus*.

Interestingly, both carvacrol and cinnamaldehyde exhibited clear antimicrobial activity against both antibiotic-resistant and non-resistant *Campylobacter jejuni* strains, at concentrations ≥ 0.1%

(Ravishankar *et al.,* 2008). Furthermore, in the studies of Inouye *et al.* (2001), Krist *et al.* (2007), Lopez *et al.* (2007) and Burt *et al.* (2007a) it was demonstrated that carvacrol, thymol, eugenol and cinnamaldehyde were very effective antibacterial compounds in the vapour phase against respiratory tract pathogens (*Streptococcus* spp., *S. aureus*), airborne microbes, foodborne microbes and carcass contaminants, respectively. Therefore, further applications could be the use in inhalation therapies to threat respiratory infections or to disinfect carcasses. Cinnamaldehyde was also found to prevent biofilm formation by *E. coli* and *Pseudomonas* spp*.,* which was probably due to the ability of sub-inhibitory concentrations of cinnamaldehyde to interfere with the quorum sensing (Niu *et al.*, 2006). Further, cinnamaldehyde at sub-inhibitory concentrations (100 µmol/L) interfered with AI-2 based quorum sensing in various *Vibrio* spp. by decreasing the DNA-binding ability of LuxR resulting in marked phenotypic changes of the bacterium including reduced virulence and increased susceptibility to stress (Brackman *et al.,* 2008). Nostro *et al.* (2007) demonstrated that carvacrol and thymol were able to inhibit formation and to eradicate biofilm of several strains of *S. aureus* and *Staphylococcus epidermis*; however the required concentration was twofold to fourfold greater than the concentration needed for inhibition of planktonic growth. Karpanen *et al.* (2008) found that thymol efficacy against *S. epidermis* in both suspension or in biofilm was different amongst two strains of the bacterium. But up to date, evidence for quorum sensing mechanisms of bacteria in the pig intestine has not been demonstrated.

Table 2. Minimal Inhibition Concentration (MIC) and Bactericidal Activity 50% (BA50) of selected essential oils tested *in vitro* in standardised assays against various bacteria

Essential oil	Species of bacteria	MIC (approximate range; µg/mL) [1]	BA50 (approximate range; µg/mL) [1]	Reference [2]
Carvacrol	*Acrobacter butzleri*	64-128		Cervenka *et al.* (2008)
	Acrobacter cryaerophilus	64		Cervenka *et al.* (2008)
	Acrobacter skirrowi	128		Cervenka *et al.* (2008)
	Bacillus cereus	188-900		Cosentino *et al.* (1999), Pol & Smid (1999)
	Campylobacter coli	500		Kamel (2001)
	Campylobacter jejuni		110	Friedman *et al.* (2002)
	Clostridium perfringens	500		Kamel (2001)
	Enterobacter cloacae	750		Aligiannis *et al.* (2001)
	Enterobacter sakazakii	188		Lee & Jin (2008)
	Enterococcus faecalis	225		Cosentino *et al.* (1999)
	Escherichia coli	100-980	570	Cosentino *et al.* (1999), Didry *et al.* (1993), Friedman *et al.* (2002), Griffin *et al.* (1999), Helander *et al.* (1998), Kamel (2001), Kim *et al.* (1995), Olasupo *et al.* (2003), Si *et al.* (2006b), Veldhuizen *et al.* (2006)
	Haemophilus influenza	63		Didry *et al.* (1993)
	Heliobacter pylori	40		Bergonzelli *et al.* (2003)
	Klebsiella pneumoniae	250-500		Aligiannis *et al.* (2001), Didry *et al.* (1993)
	Listeria monocytogenes	375-500	860	Cosentino *et al.* (1999), Friedman *et al.* (2002), Kim *et al.* (1995), Pol & Smid (1999)
	Mycobacterium avium (3)	72		Wong *et al.* (2008)
	Pseudomonas aeruginosa	300-3900		Aligiannis *et al.* (2001), Cosentino *et al.* (1999), Cox & Markham (2007), Didry *et al.* (1993), Griffin *et al.* (1999), Lambert *et al.* (2001)
	Salmonella enterica	150-500	540	Cosentino *et al.* (1999), Friedman *et al.* (2002), Helander *et al.* (1998), Kamel (2001), Kim *et al.* (1995), Nazer *et al.* (2005), Olasupo *et al.* (2003), Si *et al.* (2006b)

	Staphylococcus aureus	100-980		Aligiannis *et al.* (2001), Cosentino *et al.* (1999), Didry *et al.* (1993), Griffin *et al.* (1999), Lambert *et al.* (2001), Nostro *et al*. (2007), Veldhuizen *et al.* (2006)
	Streptococcus pneumoniae	125		Didry *et al.* (1993)
	Yersinia enterocolitica	225		Cosentino *et al.* (1999)
Thymol	*Acrobacter butzleri*	32		Cervenka *et al.* (2008)
	Acrobacter cryaerophilus	64		Cervenka *et al.* (2008)
	Acrobacter skirrowi	>256		Cervenka *et al.* (2008)
	Bacillus cereus	450		Cosentino *et al.* (1999)
	Campylobacter jejuni		240	Friedman *et al.* (2002)
	Enterobacter sakazakii	188		Lee & Jin (2008)
	Enterococcus faecalis	225		Cosentino *et al.* (1999)
	Escherichia coli	100-1000	600	Cosentino *et al.* (1999), Didry *et al.* (1993), Falcone *et al.* (2007), Friedman *et al.* (2002), Griffin *et al.* (1999), Helander *et al.* (1998), Olasupo *et al.* (2003), Si *et al.* (2006b), Walsh *et al.* (2003)
	Haemophilus influenza	125		Didry *et al.* (1993)
	Heliobacter pylori	100		Bergonzelli *et al.* (2003)
	Klebsiella pneumoniae	500		Didry *et al.* (1993)
	Listeria monocytogenes	304-450	770	Cosentino *et al.* (1999), Falcone *et al.* (2007), Friedman *et al.* (2002)
	Prevotella nigrescens	300		Shapiro (1994)
	Pseudomonas aeruginosa	385-2000		Cosentino *et al.* (1999), Didry *et al.* (1993), Griffin *et al.* (1999), Lambert *et al.* (2001), Walsh *et al.* (2003)
	Salmonella enterica	57-500	340	Cosentino *et al.* (1999), Friedman *et al.* (2002), Helander *et al.* (1998), Karapinar & Aktug (1987), Nazer *et al.* (2005), Olasupo *et al.* (2003), Si *et al.* (2006b)
	Selenomonas artemidis	200		Shapiro (1994)
	Staphylococcus aureus	140-1000		Cosentino *et al.* (1999), Didry *et al.* (1993), Falcone *et al.* (2007), Griffin *et al.* (1999), Karapinar & Aktug (1987), Lambert *et al.* (2001), Nostro *et al*. (2007), Walsh *et al.* (2003)
	Streptococcus pneumoniae	125		Didry *et al.* (1993)
	Streptococcus sanguis	1100		Shapiro (1994)
	Treponema vincentii	300		Shapiro (1994)

	Vibrio parahaemolyticus	75		Karapinar & Aktug (1987)
	Yersinia enterocolitica	155-225		Cosentino *et al.* (1999), Falcone *et al.* (2007)
Anethole	*Bacillus subtilis*	400		Kubo *et al.* (2008)
	Campylobacter jejuni		1200	Friedman *et al.* (2002)
	Escherichia coli	>1600	>6700	Friedman *et al.* (2002), Kubo *et al.* (2008)
	Enterobacter aerogens	>1600		Kubo *et al.* (2008)
	Listeria monocytogenes		>1600	Friedman *et al.* (2002)
	Micrococcus leteus	>1600		Kubo *et al.* (2008)
	Pseudomonas aeruginosa	>1600		Kubo *et al.* (2008)
	Salmonella enterica	100	>6700	Friedman *et al.* (2002), Karapinar & Aktug (1987)
	Staphylococcus aureus	500, >1600		Karapinar & Aktug (1987), Kubo *et al.* (2008)
	Vibrio parahaemolyticus	500		Karapinar & Aktug (1987)
Eugenol	*Acrobacter butzleri*	256		Cervenka *et al.* (2008)
	Acrobacter cryaerophilus	128		Cervenka *et al.* (2008)
	Acrobacter skirrowi	256		Cervenka *et al.* (2008)
	Bacillus subtilis	800		Kubo *et al.* (2008)
	Campylobacter coli	500		Kamel (2001)
	Campylobacter jejuni		220	Friedman *et al.* (2002)
	Clostridium perfringens	500		Kamel (2001)
	Enterobacter sakazakii	821		Lee & Jin (2008)
	Escherichia coli	300-2100	1100	Friedman *et al.* (2002), Griffin *et al.* (1999), Kamel (2001), Kim *et al.* (1995), Kubo *et al.* (2008), Olasupo *et al.* (2003), Si *et al.* (2006b), Walsh *et al.* (2003)
	Heliobacter pylori	100		Bergonzelli *et al.* (2003)
	Listeria monocytogenes	>1000	810	Friedman *et al.* (2002), Kim *et al.* (1995)
	Prevotella nigrescens	1000		Shapiro (1994)
	Pseudomonas aeruginosa	600-2130		Cox & Markham (2007), Griffin *et al.* (1999), Kubo *et al.* (2008), Walsh *et al.* (2003)
	Salmonella enterica	100-500	870	Friedman *et al.* (2002), Kamel (2001), Karapinar & Aktug (1987), Kim *et al.* (1995), Nazer *et al.* (2005), Olasupo *et al.* (2003), Si *et al.* (2006b)

	Selenomonas artemidis	900		Shapiro (1994)
	Staphylococcus aureus	75-2100		Karapinar & Aktug (1987), Kubo *et al.* (2008), Griffin *et al.* (1999), Walsh *et al.* (2003)
	Streptococcus sanguis	2000		Shapiro (1994)
	Treponema vincentii	800		Shapiro (1994)
	Vibrio parahaemolyticus	50		Karapinar & Aktug (1987)
Cinnamaldehyde	*Acrobacter butzleri*	32		Cervenka *et al.* (2008)
	Acrobacter cryaerophilus	32-64		Cervenka *et al.* (2008)
	Acrobacter skirrowi	64		Cervenka *et al.* (2008)
	Campylobacter coli	1000		Kamel (2001)
	Campylobacter jejuni		28	Friedman *et al.* (2002)
	Clostridium perfringens	1000		Kamel (2001)
	Escherichia coli	250-1000	570	Didry *et al.* (1993), Friedman *et al.* (2002), Helander *et al.* (1998), Kamel (2001)
	Haempohilus influenza	31		Didry *et al.* (1993)
	Klebsiella pneumoniae	250		Didry *et al.* (1993)
	Listeria monocytogenes		190	Friedman *et al.* (2002)
	Mycobacterium avium (3)	26		Wong *et al.* (2008)
	Pseudomonas aeruginosa	<200-1400		Cox & Markham (2007), Didry *et al.* (1993)
	Salmonella enterica	400-1000	330	Friedman *et al.* (2002), Helander *et al.* (1998), Kamel (2001)
	Staphylococcus aureus	125		Didry *et al.* (1993)
	Streptococcus pneumoniae	125		Didry *et al.* (1993)

(1) In the references MICs have been reported in the units mg/mL, % (v/v), µg/mL and mmol/L; for ease of comparison these have been converted to µg/mL, whereby it was assumed that EO have the same density as water; (2) MIC was determined as MBC, minimum bactericidal concentration in Bergonzelli *et al.* (2003), Cosentino *et al.* (1999) and Si *et al.* (2006b); (3) subspecies *paratuberculosis*

2.3. Synergism of mixtures of selected EO and other compounds

Carvacrol and thymol were found to give an additive effect when tested against *S. aureus* and *P. aeruginosa* (Lambert *et al.*, 2001). Synergism between carvacrol and its biological precursor *p*-cymene has been noted when acting on *B. cereus* vegetative cells. *P*-cymene, not antimicrobial to itself, swells bacterial cell membranes to a greater extent than carvacrol does, enhancing the membrane associated action of carvacrol (Ultee *et al.*, 2000a). Using a fractional inhibitory concentration index for the evaluation of the antimicrobial effect against respiratory pathogens of mixtures, Didry *et al.* (1993) found that only the combination carvacrol + thymol out of all binary mixtures of cinnamaldehyde, carvacrol and thymol showed synergism. On the contrary, Zhou *et al.* (2007a) showed that 7 out of 12 binary combinations of these 3 compounds were acting synergistically against *S.* Typhimuruim. In addition, demonstrating synergism is a hard task, different methodologies exist, and test conditions likely also influence the results to a great extent. **To summarize, up to now hard evidence for synergism between EO components is lacking.**

Due to their strong flavour, application of these EO in food products is limited (to certain products). The use of hurdle technology to reduce concentrations and to use the advantages of combinations of preservation systems could be a solution for this problem (Ultee *et al.*, 2000b). In this respect, a lot of combinations with other (antimicrobial) compounds have been tested. Nisin, which is not active against G- bacteria, combined with carvacrol or thymol showed a synergistic effect on the viability of *B. cereus* cells (Periago *et al.*, 2001; Pol & Smid, 1999) and *L. monocytogenes* (Yamazaki *et al.*, 2004; Pol & Smid, 1999) and *Bacillus subtilus* (Ettayebi *et al.*, 2000) and combined with eugenol against *L. monocytogenes* (Yamazaki *et al.*, 2004). It can be speculated that EO might act synergistically with other bacteriocins that are produced by intestinal (endogenous or exogenous) bacteria. Cinnamaldehyde was synergistic with the antibiotic clindamycin to combat *Clostridium difficile* (Shahverdi *et al.*, 2007). In an extensive study, using *S.* Typhimurium inoculated broth media

in microplate wells, Nazer *et al.* (2005) did not find clear synergistic antimicrobial effects for binary and triple combinations of carvacrol, thymol, acetic acid, lactic acid, pyropolyphosphoric acid and citric acid. Numerous dose combinations of carvacrol and thymol with either acetic acid, citric acid and EDTA against *S.* Tyhimurium have been tested by Zhou *et al.* (2007b) as well. Their results indicate that 9 out of 22 combinations were synergistic and that there were similar synergistic effects when carvacrol and thymol were combined with acetic acid or citric acid. The synergistic effect of thymol with EDTA was stronger than that of carvacrol with EDTA; the chelator EDTA alters the outer membrane permeability. Walsh *et al.* (2003) also showed that EDTA potentiated the antimicrobial activity of thymol and eugenol against both G- and G+ bacteria. Blaszyk & Holley (1998) found that sodium citrate and monolaurin could increase the inhibitory action of eugenol against 6 organisms to various degrees. In a study of Santiesteban-Lopez *et al.* (2007) synergistic binary mixtures with fractional inhibitory concentration index < 0.6 included 100 or 200 mg/kg potassium sorbate with 50 or 100 mg/kg thymol, carvacrol or eugenol against different foodborne pathogens. To conclude, there seems to be **some evidence that these EO can act synergistically with organic acids**. The mechanism for this potential synergism is, however, not clear. It can be argued that either the organic acids potentiate the effects of EO or that the EO potentiate the detrimental effects of organic acids. EO are more undissociated and lipophilic in an acidic environment (*e.g.* caused by organic acids), which is assumed to enhance their penetration in the bacterial membrane and permeabilization of the latter, hence in support of the former hypothesis. On the other hand, in line with the latter hypothesis is that stress on intracellular pH homeostasis caused by organic acids becomes lethal when EO further destabilize membrane pumps necessary for this homeostasis.

2.4. Factors interacting with the antimicrobial efficacy

MIC values are determined by diffusion methods and in microtiter plates, using low organic matter broth media. It is well established that in more complex media (higher content of organic matter), higher doses are needed to have an equal inhibition of the bacterium. Plenty of direct and indirect evidence can be found in literature. For example; Shelef *et al.* (1984) reported that **resistance** to sage EO increased **with decrease in water content and increase in protein and fat in food**.

Varel (2002) recovered more than 75% of carvacrol and thymol in the solid fraction and the remainder in the liquid fraction of the medium in a swine waste incubation experiment, indicating that these compounds can bind to organic matter. Pol (2001) stated that proteins are a limiting factor in the antibacterial activity against *B. cereus* in milk. Protein content has also been put forward as a factor inhibiting the action of clove oil on *Salmonella* Enteritidis in diluted low-fat cheese (Smith-Palmer *et al.*, 2001). Addition and condensation reactions can be involved, resulting in less active EO in the medium. The *in vitro* effect of thymol (175 mg/L) against *S.* Typhimurium was abolished by adding bovine serum albumine (9 mg/L) to the medium (Juven *et al.*, 1994). In the beginning of the past century Sollmann (1919) already found that carvacrol precipitated proteins more actively than other compounds, including thymol. When a pig starter diet was added to the broth medium, the antibacterial effect of a mixture of carvacrol, thymol, eugenol and cinnamon oil was completely abolished (Si *et al.*, 2006a). Cinnamaldehyde is known to react with proteins as stated elsewhere. Fat is another nutrient compromising antimicrobial activity in food matrices. If the EO dissolves in the lipid phase of the matrix, there will be relatively less available to act on bacteria present in the aqueous phase (Lis-Balchin, 2003). In opposite, carbohydrates in foods do not appear to protect bacteria from the action of EO (Shelef *et al.*, 1984). Smith-Palmer *et al.* (2001) showed that the lower water content of food compared to laboratory media may hamper the progress of antibacterial agents to the target site in the bacterial cell. Gutierrez *et al.* (2008) found contradictory results concerning

the effect of protein and carbohydrates (starch). It can be stated that when predicting the antimicrobial activity of an EO, the amount and type of organic compounds in the medium should be taken in account, although the relevance and consequences of this for application in animal feed with the GIT as target site is less clear since digesta herein are liquid and thoroughly mixed.

Thymol was found to be more effective against *S. aureus* and *S.* Typhimurium under anaerobic than in aerobic conditions and in lower pH conditions (Juven *et al.*, 1994). The latter is explained by the fact that thymol is a weak acid and thereby more lipophilic in an acidic environment. Cinnamaldehyde was more inhibitory for the germination of *Alyciclobacillus acidoterrestris* spores at low pH (Bevilacqua *et al.,* 2008). The maximum water solubility according to Knobloch *et al.* (1989) for carvacrol, thymol, eugenol and cinnamaldehyde is 1.1, 1.0, 0.8 and 1.4 g/L, respectively. Generally, it is assumed that when using hydrophobic substances only molecules dissolved in the aqueous phase can interact with the bacteria (Sikkema *et al.*, 1995). Gill & Holley (2006a) found that bacterial cells in proximity of suspended droplets of the EO were fastly stained with propidium iodide. Several authors questioned if the addition of emulsifiers and/or stabilizers, in order to [1] avoid separation of the hydrophobic EO from the aqueous phase, and [2] to emulsify suspended droplets to smaller particles; could improve antibacterial efficacy. *In vitro* research with oregano oil, thyme oil, carvacrol, thymol, eugenol and cinnamaldehyde in **combination with several emulsifiers and/or stabilizers** (agar, lecithine, carrageenan, Surfynol 465W, Surfynol 485W, gums, methylcellulose …) **gave inconsistent results** (Burt & Reinders, 2003; Burt *et al.*, 2005; Gaysinsky *et al.*, 2005a; Gaysinsky *et al.*, 2005b and Si *et al.*, 2006a). Burt & Reinders (2003) assumed that the reduced antibacterial activity of oregano and thyme oil against *E. coli* O157:H7 in the presence of lecithine could be explained by the fact that the emulsifier orientated itself between the oil micelles and the aqueous phase and thereby physically hindering the interaction between the compound and the bacterium or that, as it is an additional source of phospholipids, neutralized the action on the bacterial membranes.

All the former observations were done in 'closed' systems (for example food matrices). However the **GIT is a dynamic and open system** and therefore the conclusions made above should be taken with care when applied to animals. Indeed, the GIT is characterised by a (continuous) supply/transit of feed and endogeneous secretions and an intense interaction between digesta, gut physiology and gut microbiota.

2.5. Tolerance and resistance of bacteria against EO

Various studies highlighted the potential for **induced bacterial tolerance in the presence of sub-lethal doses** of several EO. Adaptation of the foodborne pathogen *B. cereus* to carvacrol after *in vitro* growth in the presence of non-lethal concentrations (60 µg/mL) was observed (Ultee *et al.*, 2000a). The mechanism behind this induced tolerance was a lowering of the bacterial membrane fluidity due to a change in the fatty acid and head-group composition of the phospholipids. Di Pasqua *et al.* (2006) found similar results for *E. coli* O157:H7 in the presence of cinnamaldehyde and for *S.* Typhimurium in the presence of carvacrol and eugenol. Various *Heliobacter pylori* strains did not acquire resistance to eugenol and cinnamaldehyde at sub-inhibitory concentrations even after 10 passages, in contrast to amoxicillin and claritromycin (Ali *et al.*, 2005). Pumbwe *et al.* (2007) found that, among other compounds, pre-treated with sub-lethal concentrations, cinnamaldehyde could induce multiple antibiotic resistance in *Bacteriodes fragilis*. Cardozo *et al.* (2004) found that in continuous ruminal *in vitro* incubations, cinnamon, anise and oregano extracts (all extracts at 7.5 mg/kg dry matter) affected the molar proportion of acetate, propionate and butyrate from day 2 to 6, but these differences disappeared after day 6. Similarly, in a pig caecal continuous culture the antimicrobial effects of 500 mg/L carvacrol on all microbial groups and *S.* Typhimurium MB2184 was not seen anymore 5 days after the addition of the EO (Report, 2006). In both cases it seems that the microbiota can adapt to the addition of EO. However, it is generally accepted that it takes at least two to three weeks for the anaerobic ruminal or caecal microbial community to establish a new

equilibrium following altered conditions. So, results from these investigations have to be interpreted with caution.

Evidence points at possible adaptation mechanisms by bacteria, but this has not yet been clearly demonstrated for complex bacterial communities. Nevertheless, no doubt that this issue is of **major importance regarding the efficacy and safety for use of these EO**, and further research is urged in this area**.**

2.6. In vitro antimicrobial activity against bacteria in the GIT of pigs

Table 2 shows data on the activity of the selected EO against a variety of bacteria in standardised assays. However, if one is considering the use of the compounds in animal feeds aiming at a modulation of the GIT bacteria, the relevance of these data becomes less obvious. Indeed, physico-chemical conditions along the GIT of pigs vary considerably and are hardly comparable with the conditions in these standardised methods. In addition, the EO have been tested mostly against pure cultures while the microbial ecology in the pig gut is characterised by a high diversity and a vulnerable stability. Therefore, one should test the effects of EO under gut-like conditions and against a mixed microbial gut population. ***In vitro*** **models, simulating the gut fermentation** and *in vivo* experiments should give more reliable data.

Several reports present data on the antimicrobial activity of these EO in *in vitro* simulations of the fermentation prevailing in the pig gut, whether carried out as batch incubations or in continuous cultures. In a 24 h batch caecal fermentation, the number of Enterobacteriaceae and gas production was lowered with 24 and 21%, respectively, by 400 mg/L carvacrol (Piva *et al.*, 2002). Si *et al.* (2006b) carried out 6 h caecal incubations and tested the effect of 300 mL/L carvacrol, 400 mL/L eugenol, 100 mL/L cinnamon oil and 200 mg/L thymol on the endogenous bacteria and introduced *E.*

coli O157:H7. All tested oils reduced *E. coli* O157:H7 counts by at least 2.5 $\log_{10}$ CFU/mL, *E. coli* counts by 1.25 $\log_{10}$ CFU/mL, and coliforms by 1.25 $\log_{10}$ CFU/mL. Only thymol reduced the number of lactobacilli (> 1 $\log_{10}$ CFU/mL). Manzanilla (2005) performed a series of gas production measurements in a closed *in vitro* system with pig intestinal contents. The results are summarized in Table 3. The **dose range needed for antimicrobial activity of the EO in these incubations falls within (Si *et al.*, 2006b) or is much higher (Manzanilla, 2005) than the range of MIC values** from Table 2.

Caco-2 cells and enteroinvasive *E. coli* EcK 262/75 were co-cultured to test the effectiveness of some EO in reducing the negative effects of the strain on cell viability (Dusan *et al.*, 2006). Carvacrol, thymol and eugenol at 55.6, 25.5 and 85.4 mg/L were beneficial, while at the higher dose (274.9, 120.2 and 410.5 mg/L, respectively) the EO, except for carvacrol, led to more damage of the cells (due to cytotoxicity of the EO). Roselli *et al.* (2007) did not find any protective effect of carvacrol and cinnamaldehyde against IPEC-1 membrane damage caused by enterotoxigenic *E. coli* K88.

The EO can interfere with the adhesion of gut pathogens. The aggregation of a pig *E. coli* strain was reduced in a Salt Aggregation Test in the presence of cinnamon and thyme oil (Kamel, 2001). Percentage aggregation is negatively related to the hydrophobicity of the bacterium and the latter is assumed to be indicative to the ability to adhere to mucosal cells of the host. Dal Sasso *et al.* (2006) found that thymol at sub-MIC levels, diminished the adherence of *E. coli* and *S. aureus* to human vaginal epithelial cells. Moreover, the haemaglutinaton of *E. coli* was abolished.

More recently, a great deal of effort has been made to investigate the effects of these EO on ruminal fermentation patterns (for reviews see Benchaar *et al.,* 2008 and Calsamiglia *et al.,* 2007).

Table 3. Effect of carvacrol and cinnamaldehyde (mg/kg fresh weight) on gas production measurements and bacterial counts (only B) in a closed *in vitro* incubation model with pig intestinal contents (after Manzanilla, 2005) [3]

Treatment	Stomach		Jejunum				Caecum
	A [1]	B [2]	A	B			B
	GP	LB	GP	GP	EB	LB	GP
Carvacrol							
100	0		0				
500				0	0	0	
1000	0	0	+	+			0
2000				++			0/+
3000				++	0	+	+
4000		+					++
10000	++		++				
Cinnamaldehyde							
100	0		0				
500				+	0	+	
1000	+	0	+	++			0
2000				++			+
3000				++	+	+	+
5000		+					++
10000	+		++				

[1] intestinal contents of 1-week old animals; [2] intestinal contents of 5-weeks old animals; [3] 0 = no reduction, + = moderate reduction, ++ = total reduction of GP = gas production, EB = Enterobacteria and LB = Lactobacilli

3. FATE OF SELECTED ESSENTIAL OILS IN MAMMALS

3.1. Pharmacokinetics and residues

Numerous data have been reported on the pharmacokinetics of EO in humans and rodents. **Carvacrol and thymol** and their metabolites were found to be **rapidly excreted in urine** after oral administration (for review see Andersen (2006) and Kohlert *et al.* (2000)). Different **oxidation products** (*e.g.* propan-1-ol derivate and thymohydroquinone) and **phase-II metabolites** (glucuronide and sulphate conjugates) were detected in rats, rabbits and humans. Unchanged compounds could be detected only in small amounts in 24-h urine. Abid *et al.* (1995) demonstrated that thymol (and many other xenobiotics) could be glucuronidated by cultured Caco-2 cells, indicating that this compound could be partly detoxified in intestinal epithelial tissue. In a clinical trial, 12 volunteers received a single dose of a preparation of a thyme extract equivalent to 1.08 mg thymol (Kohlert *et al.*, 2002). No thymol could be detected in plasma or urine, however thymol sulphate and thymol glucuronide were found in urine and only thymol sulphate was detected in plasma, suggesting the presence of UDP-glucuronyltransferase activity in kidney microsomes. Peak plasma concentrations were reached after 2.0 ± 0.8 h. In this study thymol was absorbed quickly, considerable plasma concentrations could already be detected after 20 min. Kohlert *et al.* (2000) suggested that residues in fat and muscle tissue will be limited, but literature data with pigs are scarce. In a trial with weaned piglets supplemented with thyme herb (0.1 to 1.0%), thymol was detected in the plasma (50 to 480 µg/L) but could no longer be find in plasma 3 days after the end of supplementation (Hagmuller *et al.*, 2006). Stoni *et al.* (2005) could not detect carvacrol (15.1 mg/kg feed for weaned piglets) in different tissues, including muscle and abdominal fat, but it was detected in kidney and plasma (106-171 ng/mL). In addition, a sensory panel was unable to detect a flavour/aroma difference between control and treated (500 mg/kg oregano oleoresin in diet) pork (Janz *et al.*, 2007). Oswald *et al.* (2007) showed that carvacrol and thymol could be transferred from the sow's diet (27.8 and 0.4 mg/kg,

respectively) to the milk, however the estimated recovery was very low, respectively 0.1 and 2%. Sensory characteristics of meat of lamb was not affected by diet supplementation with either 200 mg/kg carvacrol or 200 mg/kg cinnamaldehyde (Chaves *et al.*, 2008). Data on the stability in the GIT are scarce. Broudiscou *et al.* (2007) found no degradation of thymol in a 24 h *in vitro* caprine ruminal fermentation at a dose of 2000 mg/L, neither was degradation observed when the ruminal microbes were previously exposed to thymol .

In rats and mice, given single doses of 250 mg/kg BW (body weight) more than 95% of C-14 **anethole** was recovered with the majority in the 24-h urine (Bounds & Caldwell, 1996). **Eighteen urinary metabolites** were identified, the unchanged compound was not found in urine, which was in accordance to earlier work of Sangster *et al.* (1984ab, 1987) in humans. The pattern of metabolites in human urine differed only quantitatively from that seen in rodent urine. Anethole undergoes three primary oxidation pathways: O-demethylation, ω-side chain oxidation, and side chain epoxidation; followed by a variety of secondary pathways of oxidation and hydration, the products of which are extensively conjugated with sulfate, glucuronic acid, glycine, and glutathione (for further details, see Terpene Consortium, 2002). Some of the metabolites can have toxicological consequences or can result in a greater antimicrobial efficacy when applied *in vivo* compared to *in vitro* assays with the parent compound (Pauli & Schilcher, 2004). Appearance in human breast milk peaked 2 h (peak concentration; 23.3 µg/L) after a single administration of 100 mg *E*-anethole and returned to baseline after 8 h (Hausner *et al.,* 2008).

Few data are available on the pharmacokinetics of **eugenol**. Concentrations of eugenol in rat blood and plasma peaked rapidly following oral administration, but elimination half-lives in blood and plasma were long (> 14 h) (Guenette *et al.* 2007). Metabolization occurred principally in the liver via conjugation of the phenolic hydroxy group with sulphate or glucuronic acid or reduction of the double bond (Sutton *et al.*, 1985 and Guenette *et al.*, 2006).

In studies with humans and rodents it was shown that **cinnamaldehyde** was **rapidly and completely absorbed from the gut and subsequently oxidized to cinnamic acid by NAD-dependent aldehyde dehydrogenases, followed either by conjugation and excretion or by further *β*-oxidation, conjugation and excretion** (with hippuric acid as major urinary metabolite) (for review see Bickers *et al.*, 2005 and Cocchiara *et al.*, 2005 and Fig. 4). The bioavailability (AUC – area under the curve - of time-response curve in plasma after oral gavage relative to AUC after *i.v.* injection) of cinnamaldehyde was calculated to be less than 20% at dose levels 250 and 500 mg/kg BW in rats, plasma levels were less than 1 and 10 µg/mL respectively (Yuan *et al.*, 1992). In a study of Sapienza *et al.* (1993) with male F344 rats, the major urinary metabolite for doses 5 and 50 mg/kg BW (8 consecutive days) was hippuric acid, accompanied by small amounts of cinnamic and benzoic acid. At the high dose (500 mg/kg BW), benzoic acid was the major metabolite, suggesting that saturation of the glycine conjugation pathway occurs at repeated dose levels of cinnamaldehyde. After 24 h, more than 80% of the radioactivity of a single dose was recovered in the urine and less than 7% in the faeces from all groups of rats, regardless of dose level. A second pathway of cinnamaldehyde metabolism, involving conjugation with reduced glutathione to form mercapturic acid derivates was described by Peters & Caldwell (1994). Regardless of dose or species, the *β*-oxidation pathway is the predominant pathway of metabolic detoxification of cinnamaldehyde. In many studies it was shown that orally administered cinnamaldehyde was principally cleared by urinary excretion and the sum of recoveries in urine and faeces exceeded always 85%. However, it remains a matter of debate whether the compound or its metabolites can lead to appreciable residues in the animal's body. For example, 24 h after oral administration of a single dose of ^{14}C-labelled (*β*-carbon atom) cinnamaldehyde (5-500 mg/kg BW) in rats, between 0.31 and 0.90% and between 0.13 and 0.26% of radioactivity was recovered in fat and muscle respectively (Sapienza *et al.*, 1993).

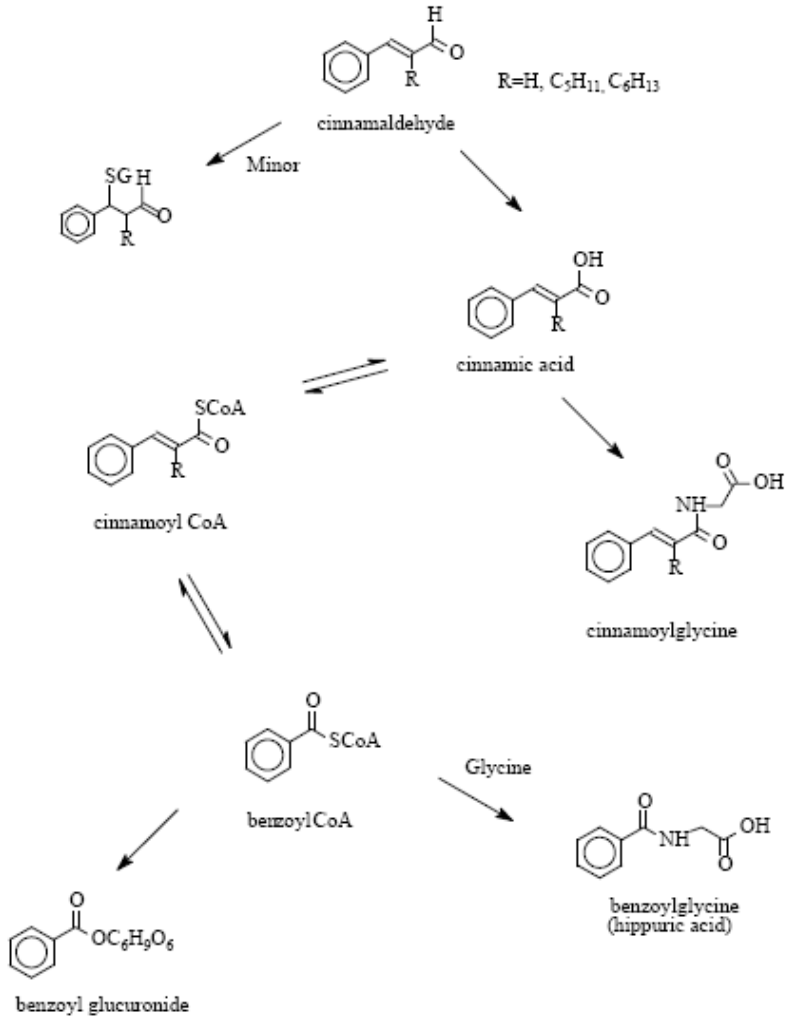


Fig. 4. Metabolism of cinnamaldehyde and derivates in mammals (Aromatic Consortium, 2005)

These data indicate rapid metabolization and clearance of these compounds/metabolites with laboratory animals and therefore **residues are primarily not anticipated**. **However, no or limited data** are available in the literature concerning the possible retention of these compounds/metabolites **in pig** tissues, expecially when higher doses of these EO are applied in the feed. So caution is recommended. Thymol is included in Annex II of Council Regulation (EEC) No 2377/90, which means that it is assumed not necessary to establish a Maximum Residue Limit (MRL) in animal species for this compound (Anonymous, 1996), indicating its presumed safety for the consumer. However, its recommended veterinary use hereby is limited to 50 mg per animal (accumulated dose); which is a very low dose, for example, in comparison with the applied doses in our *in vivo* trials reported in Chapter 4B (500 and 2000 mg/kg feed). No MRL's are available for the other EO.

3.2. Pharmacodynamics

Various body functions and tissues are responsive to EO. There is a plethora of literature data dealing with their pharmacodynamic activities in vertebrates. Most observations were done using *in vitro* models and *in vivo* confirmation is lacking many times. Some data, that have particular relevance in the context of this thesis are presented here.

3.2.1. EFFECTS ON SMOOTH, CARDIAC AND SKELETAL MUSCLE

Boskabady & Jandagh (2003) described the dose-dependent (≤ 0.52 mmol/L) **relaxant** (bronchidilatory) **effect** of carvacrol on contracted guinea-pig tracheal chains. Carvacrol showed *in vivo* cardiac hyposensitive effects in rats (Aydin *et al.*, 2007). Thymol was inhibitory for smooth (Beer *et al.*, 2007) and cardiac (Szentandrassy *et al.*, 2004) muscle contraction. On isolated blood vessels, anethole induced contraction at lower doses, mediated via opening of voltage-dependent Ca^{2+}-channels, but relaxant effects at higher concentrations (>10^{-2} mmol/L) (Soares *et al.*, 2007). In several studies it was shown that eugenol had a relaxant effect on either smooth (Nishijima *et al.*, 1999 and Leal-Cardoso *et al.*, 2002), cardiac (Damiani *et al.*, 2004) and skeletal muscle (Damiani *et al.*, 2003). The derivate methyl-eugenol showed equal antispasmodic actions (Lima *et al.*, 2000). From the studies of Leal-Cardoso *et al.* (2002), Magyar *et al.* (2002), Magyar *et al.* (2004), Nishijima *et al.* (1999), Sarkozi *et al.* (2007), Szentandrassy *et al.* (2004) and Damiani *et al.* (2004) it can be concluded that the mechanism behind the inhibitory activity of carvacrol, thymol and eugenol was largely independent of the membrane potential and participation of the enteric nervous system, but was due to interference with calcium handling. Cinnamaldehyde was found to induce the release of noradrenaline in isolated guinea-pig ileum nerve terminals in a concentration dependent manner from 5 to 50 µmol/L (Cheng *et al.*, 2000). Cinnamaldehyde can depolarize the membrane resulting in a Ca^{2+}-dependent and cAMP-related release of the neurotransmitter. This neurotransmitter is known to

induce intestinal relaxation, indirectly by the inhibition of acetylcholine release. The inhibitory action upon smooth muscle contraction has been reported for cinnamaldehyde (*i.v.* administration in rat) by Harada & Yano (1975) and on isolated rat aorta by Yanaga *et al.* (2006). So far, there is a body of evidence concerning the inhibitory effects on (smooth) muscle contraction, but it **remains questionable if these effects can affect the digestive physiology of animal (*e.g.* gut motility)** or other body functions. No data are available yet for pig.

3.2.2. ANTIOXIDANT PROPERTIES

The **phenolic compounds carvacrol, thymol and eugenol have shown dose-dependent antioxidant properties** in various *in vitro* systems (Table 4). It was stated that EO mainly owe their antioxidant activity to the H-donating capacity (Aeschbach *et al.*, 1994). The H-donating capacities can be reasonably predicted from comparisons between bond dissociation energies of their most labile H-atoms. Surprisingly, the most labile H-atom of thymol, carvacrol and eugenol was the H-atom of the isopropyl or allyl group and not the phenolic H-atom (Chemat *et al*., 2007). From Table 4 it can be noted that there is some discrepancy for data in DPPH-scavenging (diphenylpicryl hydrazyl radical) test and inhibition of lipid peroxidation (CDi - conjugated diene method). Normally, good inhibitors of lipid peroxidation must rapidly scavenge the lipid peroxyl radicals ($LOO^{\bullet}$) and this is reflected at least by a substantial ability to reduce the much less reactive DPPH radical. Here, data indicate that these compounds inhibit lipid peroxidation somewhat less efficiently than could be anticipated from their strong DPPH-scavenging activities. Probably, this is due to their lipophilic character that results in a reduced access to $LOO^{\bullet}$ (Chemat *et al.,* 2007). Mastelic *et al.* (2008) constructed derivates of carvacrol, thymol and eugenol which showed better antioxidant properties than the parent compound. Table 4 aims to illustrate their distinctive antioxidant properties, as it was demonstrated in *in vitro* models.

Table 4. Antioxidant activities of selected EO in *in vitro* systems

Essential oil (reference compounds)	*In vitro* tests of antioxidant activity [1]			Reference
	DPPH	TBARS	CDi	
Carvacrol				
(BHT, α-tocopherol)	IC50; 400 (18, 8.6)	In % at 1000; 52.8 (68.6, 90)		Kulisic *et al.* (2004)
(α-tocopherol)		In % at 1000; 69.9 (93.5)	In % at 1500; 89.9 (94.8)	Ruberto & Baratta (2000)
(propyl gallate)		In % at 100; 80 (86)		Aeschbach *et al.* (1994)
(-)		In % at 400; 64		Youdim & Deans (2002)
(trolox)		IC50; 10.5 (59.0)		Prieto *et al.* (2007)
(α-tocopherol)		In % at 1000; 52.8 (90.0)		Radonic & Milos (2003)
Thymol				
(BHT, α-tocopherol)	IC50; 500 (18, 8.6)	In % at 1000; 41.5 (68.6, 90)		Kulisic *et al.* (2004)
(α-tocopherol)		In % at 1000; 55.7 (93.5)	In % at 1500; 91.7 (94.8)	Ruberto & Baratta (2000)
(propyl gallate)		In % at 100; 82 (86)		Aeschbach *et al.* (1994)
(-)		In % at 400; 73		Youdim & Deans (2002)
(trolox)		IC50; 6.6 (59.0)		Prieto *et al.* (2007)
(α-tocopherol)		In % at 1000; 52.8 (90.0)		Radonic & Milos (2003)
Eugenol				
(α-tocopherol)		In % at 1000; 81.2 (93.5)	In % at 1500; 93.6 (94.8)	Ruberto & Baratta (2000)
(-)		IC50; 100		Dorman *et al.* (2000)
(trolox)		IC50; 13.1 (-)		Ito *et al.* (2005)
(trolox)		IC50; 2.2 (59.0)		Chericoni *et al*. (2005)
(-)		IC50; 1.85 (Fe^{2+})		Priyadarsini *et al.* (1998)
(-)		IC50; 12.2 (Fe^{3+})		Priyadarsini *et al.* (1998)
(α-tocopherol)	IC50; >130 (34.4)	In % at 10.3; 43 (97)		Ogata (2008)
Anethole				
(α-tocopherol)		In % at 1000; 41.9 (93.5)		Ruberto & Baratta (2000)

[1] DPPH test expressed as either percentage of DPPH reduced (%) at a defined concentration (mg/L) or as IC50 value (mg/L); TBARS method, values are percentage of antioxidant index (%) at a defined concentration (mg/L) or as IC50 value (mg/L); CDi, conjugated diene method expressed as percentage of inhibition of lipid peroxidation (control without added antioxidant=0%) (%) at a defined concentration (mg/L)

In vivo, thymol (42.5 mg/kg BW) was beneficial for the ageing rat brain as it maintained significant higher antioxidant enzyme activities (superoxide dismutase and gluthathione peroxidase) and total antioxidant status (Youdim & Deans, 2000). The proportion of 22:6n-3 (DHA) in brain phospholipids was also higher in the supplemented rats compared to control. It is unclear whether this can be related to retention of this compound or metabolites in the respective tissue and a biological activity thereof. Eugenol reduced TBARS, increased GSH (reduced gluthatione) and L-ascorbate, but did not affect catalase, gluthatione peroxidase and SOD (superoxide dismutase) in the striatum of mouse (Kabuto *et al.*, 2007). After 7 and 14 days administration of carvacrol (30 and 60 mg/kg/day and 15 and 30 mg/kg/day respectively) to the drinking water of rats, it was found that DNA isolated from hepatocytes and testicular cells of treated animals was significantly more resistant to the damaging effects of hydrogen peroxide than DNA of control animals (Slamenova *et al.,* 2008). The gastro-protective effect of eugenol against indomethacin-induced ulcers in rats was partially attributed to its radical scavenging properties (Morsy & Fouad, 2008). An increase in intracellular GSH and GST (gluthatione S-transferase) was attributed to anethole (cited by Chainy *et al.* (2000)) and the same compound reduced TNF (tumor necrosis factor) induced lipid peroxidation and ROS (reactive oxygen species) generation (Chainy *et al.*, 2000). Cinnamaldehyde showed no antioxidant activity in a lipid peroxidation model (Chericoni *et al.,* 2005). In contrast Gowder & Devaraj (2006) found a dose-dependent effect of this compound on antioxidative status of rat kidney. It can be concluded that these EO are promising tools to alleviate diseases in which the uncontrolled proliferation of free radicals is very damaging, although the balance between an anti- and pro-oxidant effect (likely dose-dependent) by these EO has been debated by several authors (Fujisawa *et al.,* 2002 and Bakkali *et al.,* 2008).

Related to this thesis, it has not been shown that these EO can protect the intestinal mucosa from oxidative damage. If so, it could be of great benefit for fast growing animals. EO with antioxidant activity could also be used indirectly as feed additives, in order to improve quality and shelf-life of

meat. However, as stated above, residues (and thus retention in meat) are primarily not anticipated or not fully proven. Hence, Vichi *et al.* (2001) found that back fat of pigs fed oregano extracts did not show a higher antioxidant activity than that of control animals. A tendency towards reduction of lipid oxidation was noted in oregano-fed pork (500 mg/kg oregano oleoresin in diet) (Janz *et al.,* 2007).

3.2.3. EFFECTS ON THE IMMUNE SYSTEM

Carvacrol and eugenol triggered dose-dependently the Ca^{2+} mobilization in Jurkat T-cells and THP1 monocytic cells (Chan *et al.*, 2005). As intracellular Ca^{2+} mobilization is an important factor in the signalling ways for the recruitment and activation of blood monocytes and therefore the control of inflammation, both compounds can aid the immune response. Carvacrol, thymol and related compounds found in *Nigella sativa* seeds were evaluated for their effect on COX-1 (cyclooxygenase) and COX-2 activity (Marsik *et al.*, 2005; Marsik *et al.*, 2006; Landa *et al.,* 2009). Carvacrol and thymol showed non-selective inhibition of both enzyme isoforms, with an inhibitory activity that was comparable to that of indomethacin. Thymoquinone and thymohydroquinone exhibited the strongest inhibitory effect on COX-2. Notably, the latter compound was also a phase-I metabolite of thymol found in rat plasma (Austgulen *et al.*, 1987). Previously, it was demonstrated that thymol inhibited prostaglandin synthesis (cited by Anonymous, 2000). On the contrary, Huss *et al.* (2002) found no COX-2 inhibition by thymol. Braga *et al.* (2006) showed that thymol inhibited the release of elastase by human PML in a dose-dependent manner, which was correlated to the inhibition of calcium mobilization. Eugenol in rat diets (1700 mg/kg) reduced carrageenan-induced oedema (inflammation) in foot pads (Reddy *et al.*, 2004). Prasad *et al.* (2004) showed that eugenol (IC50, 26 µmol/L) and cinnamaldehyde (35 µmol/L) inhibited 5-lipoxygenase in PML, the key enzyme involved in biosynthesis of leukotrienes which has been implicated in several inflammatory diseases in humans. Both phenylpropenes are also potent inhibitors of COX-2 activity (IC50, 125 and 245 µmol/L for eugenol and cinnamaldehyde respectively) (Huss *et al.*, 2002), but for eugenol this was contrary to

Murakami *et al.* (2007) and also eugenol did not inhibit COX-2 gene expression (Okada *et al.*, 2005). Concerning cinnamaldehyde, Guo *et al.* (2006) demonstrated that cinnamaldehyde reduced, in a dose-dependent manner, IL-1β-induced (interleukin 1 beta) COX-2 activity, but not IL-1β-induced COX-2 mRNA expression, and consequently inhibits production of PGE_2 (prostaglandin E2) in cultured rat cerebral microvascular endothelial cells. Anethole and eugenol inhibited TNF induced NF-κB (nuclear factor kappa-light-chain-enhancer) activation (Chainy *et al.*, 2000). Eugenol was shown to block the release of the bone resorbing mediators, including IL-1β, TNFα, and PGE_2 from LPS (lipopolysaccharides) stimulated macrophages (Lee *et al.*, 2007b). Cinnamaldehyde was able to inhibit LPS-induced DNA binding activity of NF-κB in addition to NF-κB transcriptional activity (Reddy *et al.*, 2004) and age related NF-κB activity (Kim *et al.*, 2007). NF-κB is a transcription factor that regulates the expression of inflammatory and immune genes. Youn *et al.* (2008) found that cinnamaldehyde suppressed the oligomerization of Toll-like receptor 4 induced by LPS, resulting in a downregulation of NF-κB activation. Further, cinnamaldehyde has been reported to inhibit activation of proliferation of T cells and to modulate their differentation (Koh *et al.*, 1998) and to inhibit pro-inflammatory cytokine secretion from monocytes/macrophages (Chao *et al.*, 2008). Taking together, **these compounds** show to some extent **anti-inflammatory/anti-pyretic** (and anti-carcinogenic) **properties**. Most of the effects, especially for cinnamaldehyde, are mediated by modulation of intracellular signalling pathways. Their potential and relevance in practical pig raising are yet to be established. Since immune response activation represents a great deal of the animal's energy budget (Klasing *et al.,* 1987; Klasing, 2004); their properties to downregulate the inflammatory responses could be of benefit to healthy animals (for example in high health status herds) under conditions of exposure to low level immune stimulation (for example by commensal gut microbiota; without the presense of a serious virulent pathogen) or in cases of excessive inflammation wherein this reaction is not appropriate in defending the menacing threat (for example coccidiosis; Klasing, 2007).

3.2.4. EFFECTS ON TRANSIENT RECEPTOR POTENTIAL CHANNELS

Among the five senses, the sense of touch (mechanical forces, chemical stimuli and temperature) is one of the most varied and least understood. The distinct touch stimuli are sensed by dorsal root ganglia and trigeminal neurons in the soma and head, respectively. These neurons convey information about the environment through specialized neuritis that extend from cell bodies. The transient receptor potential A1 (TRPA1) is member of the well-studied thermosensitive TRP channels that are present at the end of these extensions. TRPA1 is a Ca^{2+}-permeable non-selective cation channel that depolarizes the plasma membrane and causes Ca^{2+} influx. TRPA1 is activated by noxious cold temperature and several irritant synthetic and natural compounds like mustard oil and garlic, which induces a burning sensation. **Cinnamaldehyde** is also a very potent, but selective **agonist of TRPA1** through covalent modification of N-terminal cysteine residues (Bandell *et al.*, 2004). It induces a cold-sensation and was found to elicit nociceptive behaviour in mice (Bandell *et al.*, 2004) and to evoke significant spontaneous pain, heat and mechanical hyperalgesia, cold hypoalgesia and a neurogenic axon reflex erythema by applying on the forearm in study participants (Namer *et al.,* 2005). Cinnamaldehyde has been shown to provoke a heat sensation and burning pain, as exemplified on the human tongue (Albin *et al.*, 2008). Basically, cinnamaldehyde causes a burning sensation, and whether the same stimulus is interpreted as hot, cold or otherwise depends on the activity of other TRP channels and neurons. For example, TRPA1 is invariably co-expressed with TRPV1 in sensory neurons. Activation results in burning heat, because TRPA1 causes a burning sensation and TRPV1 is a heat sensitive channel. Thymol (Lee *et al.*, 2007a) and carvacrol (Xu *et al.,* 2006) were also found to be activators of TRPA1 (concentration-dependent, reversible and rapidly desensitized) and the activity of thymol was blocked by camphor. Camphor and menthol are known blockers of TRPA1, but activate other TRP channels (Macpherson *et al.*, 2006). More recently, Macpherson *et al.* (2007), Andersson *et al.* (2008) and Cruz-Orengo *et al.* (2008) found evidence that TRPA1 displays broad specificity to endogenous molecules produced during tissue damage and

oxidative stress and that are involved in anti-inflammatory processes. It is questioned if TRPA1 can function as a central chemical 'hub' nocisensor. TRPV3 is a warm-sensitive Ca^{2+} cation channel and is highly expressed in skin, tongue and nose. It was found to be a target for carvacrol, thymol and eugenol (Xu *et al.,* 2006; Vogt-Eisele *et al.*, 2007). Hereby, they elicit a warm sensation. **Activation of TRP-channels can help explain the pungent, irritant and aversive properties of these compounds.** Besides, recently a high number of publications on TRP functioning appeared showing their physiological role beyond their involment in the sense of touch.

3.2.5. EFFECTS ON ABSORPTIVE, SECRETORY AND BARRIER FUNCTION OF EPITHELIAL TISSUE

Kreydiyyeh *et al.* (2000) showed that **eugenol and cinnamaldehyde were strong inhibitors of the *in vitro* activity of** rat basolateral jejunal and kidney **Na^+-K^+-ATPase**, similarly to the action of cinnamon and clove oil (not for aniseed oil). In a rat perfusion study, they also found that the L-alanine absorption was reduced significantly by 850 mg/L eugenol and 1000 mg/L cinnamaldehyde. This reduction was attributed to the inhibition of basolateral Na^+-K^+-ATPase, the driving force for secondary active transport. It also includes that these compounds can permeate the apical membrane and reach the serosal side. The opposite was found for aniseed oil. Kreydiyyeh *et al.* (2003) demonstrated in a rat perfusion study that **aniseed oil** (*P. anisium* L.; 76.7% *E*-anethole; 0.05% of oil in first perfusate; in second perfusate 180 µmol/L glucose was added) significantly **increased jejunal glucose absorption** and this was in line with the stimulation of the Na^+-K+-ATPase activity in jejunal homogenates. Anethole also reduced urine output, the Na^+-K^+-ATPase activity in kidney homogenates was elevated. Boudry & Perrier (2008) found a dose-dependent (0-0.1 mmol/L) increase in Isc (short-circuit current) after addition of thymol and cinnamaldehyde in the Ussing chamber model. An increase in Isc means that these compounds induced transport of ions. They also found that the ionic basis was different; thymol induced Cl^- and HCO_3^- secretion via a nervous

pathway, likely by nicotinic receptor stimulation and cinnamaldehyde induced only HCO_3^- secretion, probably by direct activation of epithelial nicotinic receptors.

3.2.6. OTHER EFFECTS

Mice, fed thymol and carvacrol (200 mg/kg BW) for 7 successive days were found to have higher levels of phase-I (*e.g*. GST) and phase-II enzymes, meaning that these compounds are bi-functional inducers of xenobiotic metabolism (Sasaki *et al.*, 2005). Carvacrol has been investigated for its application in cancer therapy due to its inhibitory effects on different cancer cell types (Chan *et al.*, 2005) and in the prevention of cancer due to its anti-mutagenic activity (Ipek *et al.*, 2005). Thymol inhibited bone resorption by a direct inhibitory effect on osteoclast activity (Mühlbuer *et al.*, 2003). Carvacrol and thymol have inhibitory properties against acetylcholinesterase; carvacrol was ten times more potent than thymol (Jukic *et al.,* 2007; Orhan *et al*., 2008). Inhibition of this enzyme leads to a prolonged activation of cholinergic receptors. Priestley *et al.* (2003) showed that thymol potentiates $GABA_A$ (gamma-aminobutyric acid) receptors. Many biological actions of eugenol have been reported. For example, eugenol induces central nervous system effects in mammals, such as hypothermia, a decrease in spontaneous motor activity, and anticonvulsant and general anaesthetic effects (cited by Leal-Cardoso *et al.*, (2002)). Eugenol is a potent depressant of peripheral nervous activity and excitation–contraction coupling in skeletal muscle. At concentrations of 0.3 mmol/L or greater, both eugenol and cinnamaldehyde stimulated the activity of mitochondrial F_0F_1-ATPase, further they reduced the mitochondrial membrane potential and inhibited NADH oxidase, which would lower ATP levels and ultimately may influence cell function and growth (Kreydiyyeh *et al.*, 2000). The authors assumed that eugenol, as it is a lipophilic weak acid, may release a proton into the matrix, thus dissipating the gradient across the membrane and turning the mitochondrial ATP synthase enzyme, into an ATPase. On the other hand, a number of *in vitro* and *in vivo* experiments have demonstrated that cinnamaldehyde targets the thiols of cysteine residues on protein molecules.

For cinnamaldehyde, several biological activities such as peripheral vasodilatory, anti-platelet-aggregration, antitumor, antifungal, cytotoxic and mutagenic/anti-mutagenic activities were reported (cited by Ka *et al.* (2003) and Huang *et al.* (2007)). Subash Babu *et al.* (2007) showed that cinnamaldehyde had dose-dependent (up to 20 mg/kg BW) hypoglycaemic and hypolipidemic effects in diabetic rats and that this was caused by a stimulation of insulin secretion of pancreatic β-cells. Already in 1998, Imparl-Radosevich *et al.* (1998) have found that cinnamon aqueous extracts improved insulin receptor function by activating insulin receptor kinase and inhibiting insulin receptor phosphates, leading to increased insulin sensitiviy. Later research showed that polyphenols were mainly responsible for this effect (*e.g.* Anderson *et al.,* 2004). The use cinnamon oil for relief of diabetic complications is recommended. In addition, cinnamaldehyde (not eugenol) inhibited the activity of rat lens aldose reductase and consequently the conversion of glucose to sorbitol (Lee, 2002). Intracellular accumulation of the latter compound has been implicated in the chronic complications of diabetes (*e.g.* cataract). However, reports from clinical trials gave conflicting results and a recent meta-analysis of several clinical trials showed that cinnamon did not alter glycosylated haemoglobin, fasting blood glucose or lipid parameters in patients with type 1 or type 2 diabetes (Baker *et al.,* 2008).

3.3. Toxicity

Acute oral toxicity (LD50 – lethal dose 50%) of carvacrol and thymol have been found to be 810 and 640-1800 mg/kg BW for rodents respectively, 100 and 750 mg/kg BW for rabbits and 250 mg/kg BW for cat (only thymol) (toxicity reviewed by Andersen (2006)). Cytotoxic effects for intestinal tissue have been reported as well. Carvacrol, thymol and eugenol at 55.6, 25.5 and 85.4 mg/L had no cytotoxic effects after 24 h against differentiated Caco-2 cells, while at the higher dose (274.9, 120.2 and 410.5 mg/L, respectively) the EO, except for thymol led, to general damage of the cells and loss of monolayer adhesion (Dusan *et al.*, 2006). Roselli *et al.* (2007) found that the highest non-toxic

concentration of carvacrol towards pig intestinal IPEC-1 cells was 750 mg/L (assessed by cell permeability), while more recently, Bimczok *et al.* (2008) found that the IC50 of carvacrol towards the same cell line amounted to 78.9 mg/L (MTT assay). Carvacrol caused apoptosis in porcine lymphocyte cultures (IC50; 27.3 mg/L) as determined by annexin V-binding and caspase-3 activation (Bimczok *et al.*, 2008). Apoptosis could be a result of the effect of EO on mitochondrial membranes resulting in an impairment of the electron chain reactions and a pro-oxidant activity; as proposed by Bakkali *et al.* (2008). Acute oral LD50 for anethole was between 2090 and 3050 mg/kg BW in rodents (MSDS nr. A8638, Sigma-Aldrich, Bornem, Belgium). The determined NOEL (no observable effect level) of 300 mg/kg BW for anethole exceeds by far the estimated daily human intake in Europe (0.4 µg/kg BW), when used as a flavouring agent (Anonymous, 2004). Eugenol has a low acute oral toxicity, with a LD50 value ranging from 1930 to 3000 mg/kg BW (cited by Leal-Cardoso *et al.* (2002)). A NOEL of 500 mg/kg BW in rats was determined (Steele, 1997).

Oral acute LD50 for cinnamaldehyde in rodents was between 1160 and 2220 mg/kg BW, demonstrating that the oral acute toxicity of this substance is also rather low (Bickers *et al.*, 2005). The highest non-toxic concentration towards pig intestinal IPEC-1 cells (assessed by cell permeability) for cinnamaldehyde was 660 mg/L (Roselli *et al.*, 2007). Further details on toxicological studies with cinnamaldehyde are reviewed by Bickers *et al.* (2005) and Cocchiara *et al.* (2005). In summary; in oral chronic studies, NOAEL (no observable adverse effect level) were identified as 200 and 550 mg/kg BW in rats and mice respectively; this compound does not pose a risk for mutagenic and genotoxic activity, **but cinnamaldehyde is a skin sensitizer** in humans with a NOEL for induction of sensitization at 0.5%. Transformation of skin proteins into immunogenic entities through Michael addition reactions are thought to be responsible for the skin hypersensitizing reactivity. Surprisingly, this hypersensitivity was abolished in the presence of eugenol (quenching effect) (Basketter, 2000). Interestingly, Boyland & Chasseaud (1970), Raveendran *et al.* (1993) and Janzowski *et al.* (2003) showed that cinnamaldehyde was able to deplete GSH in rat liver *in vivo* and

in Chinese hamster V79 cells, respectively. Raveendran *et al.* (1993) and Gowder & Devaraj (2006) reported further effects of cinnamaldehyde (73.5 mg/kg BW, 30-90 days) on the rat liver and kidney antioxidant status and they concluded that cinnamaldehyde acted as a pro-oxidant. These results give evidence for cinnamaldehyde-mediated oxidative stress possibly contributing to cytotoxic/genotoxic cell damage. Additionally, cinnamaldehyde can inhibit the activity of enzymes involved in antioxidant defence, *e.g.* Van Iersel *et al.* (1997) found that cinnamaldehyde inhibited GST π-1 activity (but this occurred at 2.5 mmol/L and was not complete and reversible) and Vander Jagt *et al.* (1997) demonstrated that this compound inhibited gluthation reductase in a cell-free system. These biological functions help explain why cinnamaldehyde can be used as an anticancer agent. Depletion of GSH renders the cell more sensitive to apoptosis and therefore inhibits tumor cell proliferation. Ka *et al.* (2003) demonstrated that cinnamaldehyde induced apoptosis through a reactive oxygen species- and caspase-dependent mechanism, which was abolished with pre-treatment with vitamin E (Wu *et al.*, 2004). Further, cinnamaldehyde has been reported to have antitumor activity mediated by the inhibition of farnesyl protein transferase activity *in vitro* (Koh *et al.*, 1998).

Concluding, acute oral toxicity (cited LD50 values) for these compounds is variable according to species tested, but in most cases rather low. So it is expected that they will not pose major toxicological riks (for example with regard to current human exposure), but this has not been confirmed in pigs. Further, Stammati *et al.* (1999) reported that cinnamaldehyde, carvacrol and thymol had a variety of toxic effects at the cellular level and that these effects can be rather different between even chemically very closely related compounds, such as the isomers carvacrol and thymol. However, these authors concluded that in contrast to their cytotoxic effects, these compounds generally showed no or moderate toxicity for the animal (at least at dose levels many orders of magnitude greater than estimated levels of human exposure). The same conclusion was drawn for cinnamaldehyde by the Aromatic Consortium (2005).

4. FATE OF SELECTED ESSENTIAL OILS IN ENVIRONMENT

4.1. Distribution and fate in the environment

In vitro, carvacrol and thymol were stable in swine waste under anaerobic conditions for 62 days with 90 to 95% of the compound being recovered in the waste solids, suggesting that mixed microbial populations in swine waste do not metabolize carvacrol or thymol under anaerobic conditions (Varel, 2002). However, carvacrol and thymol were found to be biodegraded under aerobic (Chamberlain & Dagley, 1968) and anaerobic conditions (Gibson & Harwood, 2002) mainly by soil bacteria and by a diverse array of pathways. Dissipation half-lives of thymol in water and soil were respectively 16 and 5 days (Hu & Coats, 2008). Over time, a considerable volatilization loss of thymol was found in the 1 month study under the experimental conditions used. Less than 6% of thymol were detected as bound residues, and less than 3% were mineralized during the 30 day study. Anethole demonstrated ready and ultimate biodegradability (OECD Guideline 301B) with a biodegradation around 91.0% (Terpene Consortium, 2002). The soil bacterium *Arthrobacter aurescens* was able to degrade *E*-anethole, using this compound as the sole carbon source (Shimoni *et al.*, 2002). Microbial degradation of both anethole and eugenol involves oxidation of the side chain to carboxylic acid prior to hydroxylation and cleavage of the benzene ring (Shimoni *et al.*, 2002). For example, eugenol is microbially degraded to yield coniferyl alcohol, ferulic acid, vanillin and vanillic acid as intermediates (Tadasa, 1977). Cinnamaldehyde has shown to be readily biodegradable (Haarmann & Reimer, 2001). It was 100% biodegraded after 21 days in an OECD 301B test. Bock *et al.* (1988) described the reduction of cinnamaldehyde to corresponding alcohols by the fungus *Botrytis cinerea*. Microbial degradation of cinnamaldehyde is analogues to that of eugenol (Shimoni *et al.*, 2002). The transport, distribution and persistence in the environment were modelled using the EQC Fugacity Model III. Cinnamaldehyde was distributed mainly to soil and water with a short persistence in the environment (279 h) (Aromatic Consortium, 2005). Vokou & Liotiri (1999) concluded that EO are used as carbon and

energy source by ubiquitously occurring soil micro-organisms and **they would not accumulate in soil if environmental conditions favour growth of these micro-organisms**.

4.2. Ecotoxicity

Few ecotoxicological data were found in literature. EC50 (half maximum effective concentration) in acute toxicity for *Daphnia magna* and LC50 (lethal concentration) for fish *Pimephales promelas* of thymol was 1.7-3.2 and 3.2-4.2 mg/L, respectively (MSDS nr. T0501, Sigma-Aldrich, Bornem, Belgium). The acute toxicity of cinnamaldehyde in Zebra fish was determined in a 96 h semi-static test. The measured NOEC (no observable effect concentration) was 2.8 mg/L, the LC50 4.3 mg/L and the LC100 5.5 mg/L. Two tests described the acute toxicity for *Daphnia magna*. Under semi-static conditions the 48 h NOEC, EC50 and LC50 were 1.91, 3.86 and 4.22 mg/L, respectively and under static conditions EC50 equaled 11.5 mg/L. Green algae, exposed to various concentrations of cinnamaldehyde exhibited a 72 h EC of 6.87 mg/L based on average specific growth rate. Based upon the number of cells/mL, the 72 h NOEC and EC50 were 2.00 and 4.07 mg/L. Another study indicates that a 6.61 mg/L solution of cinnamaldehyde inhibits the growth of green algae by 35% after 80 h and 5% after 160 h (Aromatic Consortium, 2005).

On average, their **ecotoxicological toxicity** is in **the mg/L (ppm) range**. Since **data on environmental exposure based on current usage are lacking**, an ecological risk assessment cannot be made. Their limited persistence in the environment (see previous section) is however in favour of their environmental safety. It is not anticipated that in-feed use of these EO use will pose ecological problems. If residues are to be found in pig slurries, they are expected to be biodegraded once deposited on agricultural soils or during other manure treatment (*e.g.* composting).

5. EFFECTS OF SELECTED ESSENTIAL OILS ON GUT BACTERIA AND FUNCTIONALITY AND PIG PERFORMANCE

5.1. Effects on palatability in pigs

The sensorial qualities of a feedstuff are mainly defined by its aroma, texture and flavour (Laitat *et al*., 2003) and determine in part its palatability (reflected by ingestive response). Aroma is appreciated by olfactive (smell) and nasal sensations, while flavour perception can be ascribed to taste and olfactive and thermo-mechanical stimulation, like hot or from chemical origin (*e.g*. spicy). The pig's sense of smell is very performant and 2000 times superior to that of human. Generally, pigs accept well acidified feed, are indifferent for salt content, reject feedstuffs with a bitter taste and have a strong preference for sweet substances, in particular sucrose. It has been shown that pigs have preference for specific flavours, and which, when added to feed were able to enchance feed intake in the first weak post-weaning (not in the following weeks) (McLaughlin *et al.,* 1983). However, it is still a matter of debate to what extent flavours can positively influence feed intake and hence growth.

Due to their aromatic chemical structure and volatility, EO have a **reminiscent and characteristic (for humans mostly pleasant) aroma/flavour**. Hence, EO may affect the palatability of feedstuffs or at least the animal's preference when offered choice between either an aromatised and a non-aromatised feed. For example, they could be of great help to overcome post-weaning anorexia usually observed in commercial practice. To test palatability, **choice-feeding studies** are to be compiled (Windisch *et al.*, 2007). Indeed, increased feed intake in growth trials can simply reflect the higher consumption capacity of animals grown larger due to a better nutrient utilization compared with untreated controls. The number of choice-feeding studies with herbs/EO is very limited. They show dose-related depression of palatability in pigs fed EO from fennel and caraway (Schöne *et al.*, 2006) as well as from the herbs thyme and oregano (Jugl-Chizzola *et al.*, 2006).

5.2. Effects on gut bacteria and functionality and animal performance

Table 5 represents a **summary of published data on the effects of the EO and related products on performance in weaned piglets**. This Table may not represent all trials that have been carried out with these EO. It is believed that numerous trials, frequently without any positive effect, have probably not been published. In addition, the results of many trials carried out or (financially) supported by the industry are not accessible. Furthermore, a number of trials were not selected here, since:

[1] a huge number of 'products', often extracts or mixtures of active components, sometimes ill defined for their exact composition, have been tested;

[2] protocol details are sometimes lacking making comparison of results irrelevant, and

[3] confirmation by analysis of the intended dose of active components in the feed is rarely done.

Tests with single, pure and well defined compounds are very scarce. In the Table, treatment effects on animal performance are given as percentage difference compared to control. The reference control treatment is the negative control, which means a diet without AMGP. In some studies, a positive control was included as well, this is further described in the text below. In fact, both a negative and positive control should be used to fully understand the economical potential of these additives as alternatives.

Several trials with OEO and other plant EO have been performed as early as in the 90's. Kyriakis *et al.* (1998) and Tsinas *et al.* (1998a,b) found an increased ADG (average daily gain) and a reduced mortality, diarrhoea score and FCR (feed conversion ratio) by supplementation of OEO (Table 5). These trials were carried out on commercial pig farms with respectively a history of ETEC (enterotoxigenic *E. coli*), PE (proliferative enteropathy) and minor disease problems. Feed supplemented with 2000 mg/kg of an oregano preparation (WG Ropa, RopaFarm, The Netherlands;

containing 5% OEO of which was 81% carvacrol and 2% thymol) did not affect any of the bacterial counts and metabolites in the small and large intestine, neither it had an effect when piglets were experimentally orally infected by ETEC-O139:K82 (Gössling, 2001). The apparent digestibility of crude nutrients and the N-balance of the weaned piglets was not influenced by feeding piglets restrictively with this additive (Möller, 2001). In opposite, organic matter, crude protein and fat digestibility was improved in pigs supplemented with the same additive in a previous experiment (Günther & Bossow, 1998). In a recent trial with OEO (80 mg/kg, containing thymol and carvacrol), piglets were challenged with ETEC-O149K91+K88ac+(F4ac) at day 7 post-weaning (Jongbloed *et al.,* 2007). OEO fed pigs showed a better faeces consistency and a lower faecal *E. coli* shedding post-inoculation compared to control, while their were no differences in caecal bacterial counts at day 22 post-weaning. Further, in preliminary results of a trial by Pérez *et al.* (2007) no significant effect of OEO (150 mg/kg) on total ileal microbes post-weaning was found.

In contrast to obvious *in vitro* results by disk diffusion, 10 g/kg thyme herb (1.66 v/w EO with 39% *p*-cymene and 32% thymol) did not reduce shedding of haemolytic *E. coli*, nor it had an effect on animal performance of weaned piglets (Jugl-Chizzola *et al.*, 2005). Pigs fed fennel oil showed similar and reduced performance compared to the negative and positive (formic acid, 7.5 mg/kg and copper, 150 mg/kg) control, respectively (Schöne *et al.*, 2006) (Table 5).

In the trial of Namkung *et al.* (2004) (Table 5), ADG was reduced by 9.5% while the ileal pH, number of coliforms and lactobacilli in distal SI, intestinal morphology, white blood cell profile and plasma IL-1β, TNF-β, IgG, protein and albumin were not affected by the herbal extract mixture. PCR-DGGE (polymerase chain reaction – denaturing gradient gel electrophoresis) analysis revealed that microbiota diversity was not affected, but the number of ileal lactobacilli was increased (Gong *et al.*, 2008). A mixture containing 5% carvacrol, 3% cinnamaldehyde and 2% capsicum oleoresin in a fatty acid carrier (Xtract, Pancosma, Switzerland) dosed at 150 and 300 mg/kg had no significant

effect on animal performance, neither it had on many gut parameters like villus/crypt architecture in weaned pigs (Manzanilla *et al.*, 2004 and Nofrarias *et al.*, 2006) (Table 5). Supplementation increased weight and pH of stomach content. At 300 mg/kg it tended to increase jejunal lactobacilli and to decrease enterobacteria when determined by direct plating; these results could not be confirmed by quantitative PCR analysis of the digesta (Castillo *et al.*, 2006). Further, this supplementation reduced significantly the number of IEL (intra-epithelial lymphocytes) in jejunum and lymphocytes in ileocolic lymph nodes; however blood monocytes and the density of LPL (lamina propria lymphocytes) were increased. The higher stomach content reflects a lower gastric emptying rate and the authors argued that this was due to the direct effect of capsaicin on gastric motility. Oetting *et al.* (2006b) found a dose-dependent rise in apparent dry matter and energy digestibility when animals were supplemented with a herbal extract mixture, but this was not in line with animal performance in a growth trial (Table 5). The 2100 mg/kg treatment gave intermediate performance results between the negative and positive control (zinc bacitracin, 50 mg/kg; olanquindox, 50 mg/kg and colistin, 50 mg/kg), although it has to be stressed that in this trial growth rates were low. Histological parameters for this treatment compared to negative control were not altered, improved and worse in duodenum, jejunum and ileum respectively. Herbal treatments had no effects on microbial plate counts in SI (Oetting *et al.*, 2006a). PRC-DGGE analysis of the bacterial population showed higher similarity, due to sampling site, than to treatments applied (Pedroso *et al.*, 2005). Cluster analysis of V3 region of 16S rDNA amplicon showed that, within each small intestinal sampling site, distances within herbal treatments were smaller than compared to negative and positive controls. Piglets supplemented with a mixture of CEO (clove essential oil), eugenol, OEO and carvacrol showed similar performance as the positive control treatment (colistin, 75 mg/kg and tiamulin, 75 mg/kg), while other treatments performed less (Costa *et al.*, 2007) (Table 5).

Microencapsulated thymol did not change bacterial counts in different sections of the GIT (Piva *et al.*, 2007b) (Table 5). However, in this abstract, the applied dose was not mentioned. Thymol at a

dose of 10000 mg/kg was applied in the trial of Trevisi *et al.* (2007) (Table 5). Additionally, half of the piglets were challenged with *S.* Typhimurium at day 5 post-weaning. Thymol reduced feed intake in the first days post-weaning and thereafter, had no effect on *S.* Typhimurium excretion, neither on tissue weights, digesta pH, salivary IgA, saliva production, ATPase gene expression, gastric TNF expression, number of parietal cells for each oxyntic gland and the depth of this gland. Serum IgA and IgM was significantly increased at day 5 by the thymol addition. In a subsequent publication, a molecular analysis (PCR-DGGE of the V6-V8 region of 16S rRNA) of the jejunal microbiota was presented (Janczyk *et al.,* 2008). No effect of challenge or thymol supplementation on the bacterial diversity could be observed. Clustering of the objects revealed the presence of distinct clusters according to challenge and thymol supplementation; showing a shift in the jejunal bacterial community caused by treatment. In thymol treated animals, the G- bacterium *Actinobacillus minor* was absent and in contrast, the G- enterobacterium *Citrobacter freundii* was present in most animals fed thymol. Cinnamaldehyde, supplemented at 175 mg/kg, did not improve ADG, nor it had an effect on FCR and histology of jejunum (Andrés Elias *et al.,* 2007).

Several trials (Table 5) indicate that **OEO at a particular low dose (range between 12.5 and 345 mg phenolic compounds per kg feed) is able to significantly improve ADG and reduce FCR**. Besides, OEO was able to ameliorate faeces consistency and lower diarrheoa incidence and ETEC shedding (after inoculation), without affecting gut microbial counts. **However**, in the studies of Gollnisch & Halle (2001) and Wald *et al.* (2001) OEO (100 mg/kg) did not improve animal performance, neither did a range of **other plant EO**. Probably, this inconsistency can partially be related to the hygiene status of the animals and hence performance of the control animals. 'Antimicrobial' feed additives are expected to be more beneficial for challenged animals, housed under low hygienic conditions. **Mixtures** were tested by several groups, but firm conclusions cannot be made. Surprisingly, **in some of these trials direct plating and molecular analysis of microbes gave conflicting results**. These discrepancies can explained by the agglomeration of colonies on the

plates, the presense of a high number of viable but not culturable bacteria in the digesta, the amplification and quantification of free DNA from dead bacteria in the digesta and the multiplicity of 16S rDNA genes per genome in prokaryriotic organisms (Castillo *et al.,* 2006). **Few trials have been conducted with pure compounds.** A general observation is that **in none of these studies the total number of bacteria in the GIT was reduced**. **Effects on histo-morphological and immune parameters were mostly lacking or variable.**

Fewer trials with growing and finishing pigs have been reported compared to weaned pigs. In the trial of Tsinas *et al.* (1998b) (see above and Table 5) the pigs were reared until slaughter weight. Animal performance and health status was equally improved as in the weaner period, and prevelance of *Lawsonia intracellularis* was markedly reduced. Walter & Bilkei (2004) studied the effect 3000 mg/kg of Orepig (Oregpig GnbH, Pecs, Hungary) on the proportions of leukocyte sub-populations in growth-retarted growing-finishing pigs subject to various infectious agents. Orepig is the dried leaf and flower of *O. vulgare*, enriched with 500 g/kg cold-pressed EO of the leaf and flower of the plant, equivalent to 60 mg carvacrol and 55 mg thymol per kg. The proportion of $CD4^+$, $CD8^+$, MHC class II antigen and γδ-T cells in peripheral lymphocytes and double positive $CD4^+CD8^+$ in peripheral blood and mesenteric lymph nodes showed significantly higher values in the supplemented pigs from 4 weeks supplementation on. As well, ADG and FCR was significantly better, but it has to be mentioned that the trial was conducted with severe growth-retarded and diseased animals.

A significant reduced sow mortality and marked improved reproductive performance (significant decreased weaning-to-oestrus interval and increased farrowing rate) was observed after application of 1000 mg/kg Oregpig in the pre-farrowing diet and lactation diet (from parturition throughout lactation and from weaning to mating) (Allan & Bilkei, 2005; Amrik & Bilkei, 2004; Kis & Bilkei, 2003 and Mauch & Bilkei, 2004). However, also in these studies the performance of the control group was low compared to EU standards. Ilsley *et al.* (2003) found that sow and piglet performances

from day 15 to 21 were better with a diet supplemented with 100 mg/kg of a mixture of 5% carvacrol, 3% cinnamaldehyde and capsicum oleoresin 2% in a fatty acid carrier (Xtract, Pancosma, Switzerland) compared to control.

Varel (2002) and Varel & Miller (2004) investigated the applicability of carvacrol, thymol and eugenol to reduce odour emissions and pathogen proliferation in swine waste (here added directly to swine slurries). Carvacrol or thymol at 2500 mg/L and eugenol at 2680 mg/L completely inhibited the production of the offensive odour compounds; isobutyrate, valerate, isovalerate and cresol, and significantly reduced other SCFA and gas emissions from swine waste. Fecal coliforms were reduced from $6.3\ 10^6$ to $1.0\ 10^3$ cells per mL 2 days after treatment with carvacrol (2000 mg/L) and were not detectable within 14 days. Notably, lactate accumulated only when carvacrol, thymol or eugenol were added and not in the control treatment. As mentioned before, carvacrol and thymol were stable in swine waste under anoxic conditions for 62 days. In conclusion, carvacrol and thymol are not metabolized in anoxic swine waste and they are potentially useful in controlling odor emissions and pathogens in swine waste. Thymol (2000 mg/L) reduced *E. coli* and *S.* Typhimurium proliferation in swine slurries (Wells & Varel, 2008).

Table 5. Effect of plant extracts and essential oils and related plant extracts on production performance in weaned piglets [4]

Plant extracts and essential oils (intented dose of additive as fed; mg/kg / formulation type)	Intended dose of active ingredient(s) (mg/kg)	Basal diet	Weaning age (d) / weight (kg)	Dura-tion of trial (d)	Treatment effect (% difference from untreated control) [1]			Reference
					ADFI	ADG	FCR	
Oregano oil (Ropadiar) (500 / ICA)	OEO, 25 (81% CAR, 2% THY)	cereals, SBM	20 / 7.78	42	-2.6	+7.1*	-9.1*	Günther & Bossow (1998)
Oregano oil (Ropadiar) (500 / ICA)	OEO, 25 (81% CAR, 2% THY)	-	28 / 7.5	14	+4.3	+16.6*	-12.0*	Van Krimpen *et al.,* 2001)
Oregano oil (Ecodiar) (250 / ICA)	OEO, 12.5	-	25 / -	21	0.9	+9.4*	-7.3*	Kyriakis *et al.* (1998)
(500 / ICA)	OEO, 25	-	25 / -	21	+11.3*	+18.5*	-9.3*	Kyriakis *et al.* (1998)
Oregano oil (Ecodiar) (250 / ICA)	OEO, 12.5	-	25 / -	37	-6.9	+3.4	-9.8*	Tsinas *et al.* (1998b)
(500 / ICA)	OEO, 25	-	25 / -	37	-5.9	+5.9*	-10.7*	Tsinas *et al.* (1998b)
Oregano oil (Ecodiar) (250 / ICA)	OEO, 12.5	-	25 / -	37	-1.8	+6.6*	-7.9*	Tsinas *et al.* (1998a)
(500 / ICA)	OEO, 25	-	25 / -	37	-6.6*	+8.2*	-13.7*	Tsinas *et al.* (1998a)
Oregano (Oregpig) (1000 / -)	OEO, 500 (CAR, 60; THY, 55)	-	28 / 8.1	21	0	+2.1	-2.1*	Molnar & Bilkei (2005)
(2000 / -)	OEO, 1000 (CAR, 120; THY, 110)	-	28 / 8.1	21	+6.4*	+8.9*	-2.1*	Molnar & Bilkei (2005)
(3000 / -)	OEO, 1500 (CAR, 180; THY, 165)	-	28 / 8.1	21	+4.9*	+8.8*	-3.7*	Molnar & Bilkei (2005)
Oregano oil (500 / ICA)	OEO, 25	-	21 / 5.4	28	+1.1	-0.2	+0.8	Neill *et al.* (2006)
(1000 / ICA)	OEO, 50	-	21 / 5.4	28	+2.0	-0.8	+1.6	Neill *et al.* (2006)
(2000 / ICA)	OEO, 100	-	21 / 5.4	28	+2.4	+0.3	+0.8	Neill *et al.* (2006)
Oregano oil (Royal P.) (1500 / DC)	OEO, 112.5	corn, SBM	17 / 5.6	35	-7.1*	-6.2*	+1.2*	Ragland *et al.* (2007)
Oregano oil (Royal N.) (500 / DC)	OEO, 37.5	corn, SBM	21 / 5.2	35	-3.1*	-5.5*	+6.5*	Ragland *et al.* (2007)
(1000 / DC)	OEO, 75	corn, SBM	21 / 5.2	35	-3.8*	+1.1	-3.0*	Ragland *et al.* (2007)
(1500 / DC)	OEO, 112.5	corn, SBM	21 / 5.2	35	+4.2*	+10.7*	-3.0*	Ragland *et al.* (2007)
Oregano oil (- / -)	OEO, 100	-	- / 7	35	+3.0	+2.3	+0.7	Gollnisch & Halle (2001)
Clove oil (- / -)	CEO, 100	-	- / 7	35	+1.0	-1.5	+2.7	Gollnisch & Halle (2001)
Cassia oil (- / -)	CAEO, 100	-	- / 7	35	+4.9	+2.3	+2.7	Gollnisch & Halle (2001)
Oregano oil, clove oil (- / -)	OEO, 50; CEO, 50	-	- / 7	35	+3.0	+0.5	+2.7	Gollnisch & Halle (2001)

Oregano oil, cassia oil (- / -)	OEO, 50; CAEO, 50	-	- / 7	35	+3.7	-0.3	+4.0	Gollnisch & Halle (2001)
Clove oil, cassia oil (- / -)	CEO, 50; CAEO, 50	-	- / 7	35	+1.3	-1.8	+2.7	Gollnisch & Halle (2001)
Clove oil (- / -)	CEO, 100	cereals, SBM	- / 8.2	35	-	+6.5	-3.7	Wald *et al.* (2001)
Oregano oil (- / -)	OEO, 100	cereals, SBM	- / 8.2	35	-	+5.4	-4.9	Wald *et al.* (2001)
Thyme oil (- / -)	TEO, 100	cereals, SBM	- / 8.2	35	-	-4.3	-1.8	Wald *et al.* (2001)
Cassia oil (- / -)	CAEO, 100	cereals, SBM	- / 8.2	35	-	+0.2	-4.9	Wald *et al.* (2001)
Fennel oil (100 / -)	FEO, 100 (62.5% ANE)	cereals, SBM	21 / 7.0 [(2)]	21	+2.7	+3.9	-1.5	Schöne *et al.* (2006)
Cinnamon, thyme, oregano (7500 / ICA)	-	corn, SBM	16-19 / 4.90	28	-16.7	-9.5	-7.4	Namkung *et al.* (2004)
Carvacrol, cinnamaldehyde and capsicum oleoresin (Xtract)								
(150, ICA)	CAR, 7.5; CIN, 4.5	corn, barl., SBM	20 / 6.0 [(3)]	21	-6.9	-10.8	+3.2	Manzanilla *et al.* (2004)
(300, ICA)	CAR, 15; CIN, 9	corn, barl., SBM	20 / 6.0 [(3)]	21	-6.9	-6.4	-1.5	Manzanilla *et al.* (2004)
Herbal extract mixture (700, ME)	TEO, 46.6; CEO, 23.3; OEO, 23.3; EUG, 23.3; CAR, 23.3	corn, SBM	21 / -	35	-1.2	-3.4	+3.4	Oetting *et al.* (2006b)
(1400, ME)	TEO, 93.2; CEO, 46.6; OEO, 46.6; EUG, 46.6; CAR, 46.6	corn, SBM	21 / -	35	-9.9	-11.0	+1.1	Oetting *et al.* (2006b)
(2100, ME)	TEO, 186.4; CEO, 93.2; OEO, 93.2; EUG, 93.2; CAR, 93.2	corn, SBM	21 / -	35	+1.7	+0.6	+0.6	Oetting *et al.* (2006b)
Herbal extract mixture (- / ME)	CEO, 210; EUG, 210	corn, SBM	24 / 7.2	35	-9.8	-12.9*	+11.0	Costa *et al.* (2007)
(- / ME)	OEO, 210; CAR, 210	corn, SBM	24 / 7.2	35	-3.6	-10.8*	+8.4	Costa *et al.* (2007)
(- / ME)	CEO, 105; EUG, 105; OEO, 105; CAR, 105	corn, SBM	24 / 7.2	35	+6.0	+5.7*	+3.2	Costa *et al.* (2007)
Thymol (- / ME)	THY, -	-	22 / 6.7	42	+12.6*	-	-	Piva *et al.* (2007)
Thymol (10000 / -)	THY, 10000	-	24 / -	25	-12.9*	-7.6	-6.4*	Trevisi *et al.* (2007)

[(1)] * = significantly different compared to negative control ($P<0.05$); [(2)] experimental period started 5d post-weaning; weight at day 5 post-weaning; [(3)] experimental period started 12d post-weaning, weight at weaning and [(4)] for Abbreviations, see List of Abbreviatons.

6. LEGAL FRAMEWORK REGARDING THE USE OF EO IN ANIMAL FEEDING IN THE EU

Herbal extracts, flavours and EO now fall within the scope of **EC Regulation 1831/2003**. In general, unprocessed herbs would be regarded as feed materials and thus not in need for authorisation. Notified plant extracts or components thereof are now included in the Community Register of Feed Additives (accessed Dec. 2008, http://ec.europa.eu/food/food/animalnutrition/feedadditives/registeradditives_en.htm). This register has only informative purposes and does not replace Community legal acts. The Community legal acts concerning the authorisation of each additive entered in the Register constitute the legal basis for the placing on the market and use of the additive concerned. This means that before November 2010, a complete scientific dossier for each notified EO or component thereof shall be submitted to EFSA (European Food Safety Authority). After full evaluation by EFSA, with positive outcome and after authorisation by the Standing Committee on the Food Chain and Animal Health (SCFCAH), these EO can be used legally in the EU in animal nutrition. Full references on legal aspects can be found at http://ec.europa.eu/food/food/animalnutrition/feedadditives/index_en.htm. The actual status of the EFSA evaluation of the scientific dossier can be checked at the Register of Questions (http://registerofquestions.efsa.europa.eu/roqFrontend/questionsList.jsf).

CHAPTER 2

IN VITRO CHARACTERISATION OF THE ANTIMICROBIAL ACTIVITY OF SELECTED ESSENTIAL OIL COMPONENTS AND BINARY COMBINATIONS AGAINST THE PIG GUT FLORA

Redrafted after

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. A. (2007). *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. Livestock Science, 109, 157-160.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. A. (in press). *In vitro* characterisation of the antimicrobial activity of selected essential oil components and binary combinations against the pig gut flora. Animal Feed Science and Technology, doi:10.1016/j.anifeedsci.2009.01.004

CHAPTER 2

IN VITRO CHARACTERISATION OF THE ANTIMICROBIAL ACTIVITY OF SELECTED ESSENTIAL OIL COMPONENTS AND BINARY COMBINATIONS AGAINST THE PIG GUT FLORA

ABSTRACT

The antimicrobial activity of selected EO components against the major culturable components of the pig gut flora has been characterised by means of an *in vitro* incubation model simulating the fermentation in different sections of the pig GIT. In a first study 7 components were screened for their antimicrobial properties. Dose-response equations were established for the 4 components with the highest potential in a second study. Binary combinations were tested as well, and the interaction effects were evaluated following the isobole method. The results of both studies indicated that carvacrol, thymol, eugenol and *E*-cinnamaldehyde give opportunities to modulate the flora and fermentation pattern of the GIT of pigs. Eucalyptol, terpinen-4-ol and *E*-anethole were found not to have interesting effects on the growth of the pig gut flora. The minimum concentration for carvacrol, thymol, eugenol and *E*-cinnamaldehyde in jejunal simulations to reduce the number of total anaerobic bacteria compared to control with a probability of 99.7% was 255, 258, 223 and 56 mg/L respectively. This strong activity of *E*-cinnamaldehyde was due to its progressively increasing effect against coliform bacteria; a dose of 104 mg/L gave a reduction of 1 $\log_{10}$ CFU/mL vs. 371, 400 and 565 mg/L for carvacrol, thymol and eugenol respectively. However, *E*-cinnamaldehyde showed clearly less inhibitory activity towards lactobacilli than carvacrol and thymol. Therefore, the use of

E-cinnamaldehyde (for example 100 mg/L) and to a lesser extent eugenol could result in a shift in the microbial ecology in favour of lactic acid producing bacteria and reducing the number of coliform bacteria. Carvacrol and thymol showed very similar and non-selective antimicrobial properties. Their effect was more pronounced in acidic media and demonstrated a rapidly increasing bactericidal effect from a certain concentration on (400-500 mg/L in jejunal simulations). The inhibition of the production of total SCFA in jejunal simulations by these 4 candidates was related to their effect against coliform bacteria, however they did not alter the lactic acid and ammonia concentrations. Few combinations demonstrated synergism; most mixtures showed zero interaction or antagonism. Carvacrol + thymol (ratio ≥1) was synergistic against total anaerobic bacteria in jejunal simulations, however this effect was rather small. In caecal simulations, carvacrol, thymol and *E*-cinnamaldehyde were equally effective while eugenol had only an effect on coliforms. These data on the *in vitro* antimicrobial activities of EO components give support for a better control of the gastrointestinal bacterial community and design of alternative growth-promoters. Their *in vivo* potential is discussed as well.

KEYWORDS: *in vitro* antimicrobial activity, carvacrol, thymol, eugenol, *E*-cinnamaldehyde, pig gut flora, lactobacilli, coliforms

INTRODUCTION

For reasons of public concern and legislation (regulation EC/1831/2003 banned the use of in-feed antibiotics – AMGP - in the EU as from January 2006) there is a need for reliable alternatives for the AMGP. For decades, the use of AMGP in intensive pig production demonstrated a consistent ability to improve growth rates and feed conversion ratios (Cromwell, 2002). Their mode of action is not fully understood but there is evidence that nutritional antibiotics work in part by decreasing the overall numbers and/or specific bacterial species or their metabolic activities in the GIT (see reviews by Visek, 1978; Anderson *et al.,* 1999; Corpet, 2000; Gaskins *et al.*, 2002; Dibner & Richards, 2005 and Page, 2006). Dierick *et al.* (2002a) stated that growth promotion by in-feed antibiotics is related and proportional to the inhibition of the total microbial load and microbial metabolism in the stomach and the jejunum. A reduction of the general bacterial growth in SI and pathogen proliferation should be major targets to improve animal performance and/or health (Apajalahti & Kettunen, 2006).

Herbs, spices, plant extracts and derived products have been proposed and reviewed as alternatives for AMGP in pig raising (Doyle, 2001; Kamel, 2001; Turner *et al.*, 2001; Rodehutscord & Kluth, 2002; Lis-Balchin, 2003; Wenk, 2003; Lee *et al.*, 2004 and Westendarp, 2005). However, different modes of action (*e.g.* antioxidant, antimicrobial, coccidiostatic, anti-inflammatory, immunomodulating effects and enhancement of endogenous secretions) are described depending on the product considered. Here, research has been carried out concerning the antimicrobial activities of some specific plant-derived pure chemicals. A large quantity of chemicals of herbal origin are well known for their antimicrobial properties (for review see Cowan, 1999; Hulin *et al.*, 1998; Nychas & Skandamis, 2003 and Burt, 2004) with the EO and their components being a very interesting and promising group.

Most of the observations of antimicrobial properties of EO and their components found in literature are based on standardised broth dilution or disc and agar well diffusion assays. However, problems associated with the assessment of the antimicrobial activity by these diffusion methods are that the method in itself is highly dependent on water solubility and the ability of test components to diffuse through agar (Southwell *et al.*, 1993) and on the volatility of the oils. Turbidimetry (measurements of optical density) used in broth dilution assays detects only the upper part of growth curves (> 6 $\log_{10}$ CFU/mL), is influenced by the size of the bacterial cells, and requires calibration in order to correlate the results with viable counts obtained on agar media (plate counting) (Nychas & Skandamis, 2003). Moreover, physico-chemical conditions along the GIT of pigs vary considerably and are hardly comparable with the conditions in these standardised methods. Finally, these EO and components have been tested mostly against pure cultures while the microbial ecology in the pig gut is characterised by a high diversity and a vulnerable stability. Therefore one should test these EO and components under gut-conditions and evaluate their effects against a mixed microbial gut population.

The aim of the present work was therefore to characterise the antimicrobial activity against the main culturable components of the pig gut flora of a selected number of EO components by means of *in vitro* batch incubations simulating the fermentation in different sections of the pig GIT in order to fully understand their potential as an alternative for the AMGP. The EO components included the monoterpenes: carvacrol, thymol, eucalyptol and terpinen-4-ol and the phenylpropenes: *E*-anethole, eugenol and *E*-cinnamaldehyde. These hydrophobic components have MIC values ranging between 50 and 5000 mg/L depending on the EO component, methodology and test organism. For review on this topic the reader is directed to Burt (2004).

MATERIALS AND METHODS

In vitro simulations of the pig gut fermentation

CHEMICALS

Carvacrol (282197), thymol (T0501), eucalyptol (C80601), *E*-anethole (117870) and eugenol (E51791) were obtained from Sigma-Aldrich nv/sa (Bornem, Belgium) and *E*-cinnamaldehyde (96320) was obtained from Fluka (Bornem, Belgium). Acros Organics (Geel, Belgium) provided terpinen-4-ol (36002). According to the manufacturer, terpinen-4-ol was synthetically prepared and therefore expected to be an 1:1 mixture of both enantiomers; (+)-terpinen-4-ol and (-)-terpinen-4-ol. This was confirmed by GC-analysis using a chiral separation column (Restek Rt-βDEXsm; Bellefonte, USA). Tea tree EO was delivered by Pranarôm (Ghislenghien, Belgium) and contained 38,7% terpinen-4-ol which was by far the most important out of 58 identified components. GC-analysis using a chiral separation column showed that 65% and 35% of terpinen-4-ol was (+)-terpinen-4-ol and (-)-terpinen-4-ol, respectively.

SIMULATION OF THE PIG GUT FERMENTATION

The antimicrobial activity against the pig gut flora was evaluated *in vitro* under conditions prevailing *in vivo* in the stomach, jejunum and caecum of pigs. The *in vitro* batch incubation medium was composed of 2 g of an artificial substrate (Table 1), 20 mL of a buffer, 1 mL of a suspension of pig gut flora (inoculum) and 50 µL of a solution of the EO component in absolute ethanol.

Table 1. Composition of the artificial substrate for *in vitro* simulations of pig gastric, jejunal and caecal fermentation (mg/kg)

Ingredient	Gastric simulations	Jejunal simulations	Caecal simulations
Starch from corn [(1)]	650	650	100
Dextrose, anhydrous [(2)]	100	100	-
Casein [(3)]	100	100	100
Casein acid hydrolysate [(4)]	20	20	5
Soybean oil	30	30	10
Bile salts [(5)]	-	0.65	-
L-cysteine hydrochloride anhydrous [(6)]	-	-	6.5
Fibre			
pectine NF pure citrus medium [(7)]	20	20	105
alphacel [(8)]	40	40	550
mucine from porcine stomach, type II [(9)]	10	10	100
Premix Biosow [(10)]	30	29.35	23.50

[(1)] Sigma, S-4126, [(2)] Sigma, D-9434, [(3)] Sigma, from bovine milk, C-7078, [(4)] MP Biomedicals, LLC, 101291, [(5)] Sigma, B-8756 (50% sodium cholate and 50% sodium deoxycholate), [(6)] Sigma, C-1276, [(7)] Upjohn, item nr. 00 160825 000 5, [(8)] MP Biomedicals, LLC, 900453, [(9)] Sigma, M-2378, [(10)] Vitamex NV, Drongen; Belgium (Vit A, 400 IU/g, Vit D3, 80 IU/g, Vit E, 2400 mg/kg, Vit K, 40 mg/kg, Vit B1, 50 mg/kg, Vit B2, 150 mg/kg, Vit B3, 480 mg/kg, Vit B6, 80 mg/kg, Vit B12, 950 µg/kg, Folic acid, 32 mg/kg, Choline, 8000 mg/kg, Fe, 4800 mg/kg, Cu, 800 mg/kg, Zn, 4000 mg/kg, Mn, 3270 mg/kg, I, 45 mg/kg, Co, 45 mg/kg, Se, 15 mg/kg, antioxidants (BHT and ethoxyquin), 1500 mg/kg)

The final concentration of ethanol in the incubation medium never exceeded 2.4 mL/L. The concentration of bile salts used in the jejunal simulations is rather low but within the physiological range of bile salts occurring in pig intestinal digesta (Campbell *et al.*, 2004). The duration of the *in vitro* incubations was 3, 4 and 24 h for simulation of gastric, jejunal and caecal fermentation respectively, allowing an exponential growth of the bacteria. They were carried out in 100 mL vessels in a shaking water bath. The incubation system and parameters chosen resemble as closely as possible the *in vivo* conditions (Table 2). The medium and headspace in the 100 mL vessels for caecal simulations were gassed with a mixture of 90% N_2 + 10% CO_2 before starting the incubation and after adjusting the pH to 6.5.

Table 2. *In vitro* batch incubation conditions simulating the pig gastric, jejunal and caecal fermentation

Parameter	Gastric simulations	Jejunal simulations	Caecal simulations
pH	3	6.5	6.5 (adjusted after 12 h with 1 mmol/L NaOH)
Medium and headspace	aerobic (free air)	aerobic (free air)	anaerobic (90% N_2 + 10% CO_2)
Duration (h)	3	4	24
Shaking waterbath	37°C ; f = 90 rpm	37°C ; f = 90 rpm	39°C ; f = 90 rpm
Buffer (mL)	20, glycine 0.2 mmol/L	20, phosphate 0.1 mmol/L	20, phosphate 0.1 mmol/L
Inoculum (mL)	1	1	1
Treatments (mg/L)			
- first study	0, 20, 100 and 500	0, 10, 100 and 1000	0, 10, 100 and 1000
- second study	0, 100, 200, 300, 400 and 600	0, 100, 200, 300, 400 and 600	

INOCULUM

For practical reasons different approaches were applied for the preparation of the inoculum. For each gastric and jejunal incubation, a piglet fed a wheat-pea based diet without any antimicrobial growth-promoter and weaned for 3 to 4 weeks was euthanised and the GIT was removed. The SI was divided into three segments of similar length. For the gastric incubations the contents of the two cranial segments were quantitatively collected and centrifuged (10 min, 1500 *g*, 5°C). The supernatant that contained a suspension of the luminal flora was used as inoculum. Small intestinal flora was used as inoculum for *in vitro* gastric simulations because of the ease of collection and isolation, and the fact that the dominant luminal indigenous flora components at both sites (stomach and jejunum) are the same (Vervaeke *et al.*, 1979 and Jensen, 1998). For the jejunal incubations the content of the complete SI was collected and further processed in a similar way as for the gastric incubations. However, due to temporary unavailability of fresh flora, preservation techniques were used for the preparation of the inoculum for jejunal and caecal simulations in the first series of experiments. The

small intestinal contents of 8 slaughter pigs (ca. 105 kg BW) with known origin and feeding (commercial diet without any antimicrobial growth-promoting additive) were collected and mixed; feed particles were removed by centrifugation of these contents (10 min, 1500 *g*, 5°C); thereafter the flora was isolated by centrifugation of the supernatant (10 min, 15000 *g*, 5°C). This flora was then resuspended in a 40 g/L trehalose and 40 g/L sucrose solution and kept frozen at -80°C in 25 ml vials, according to Zayed & Roos (2004). For use as inoculum in jejunal simulations the frozen suspension was thawed, centrifuged (10 min, 15000 *g*, 5°C) and resuspended in a sterile 9 g/L NaCl-solution. In a preliminary trial we investigated the effect of this preservation technique on the bacterial groups used in this study. A slight reduction in the number of CFU/mL of the bacterial groups considered occurred. However, the ratio between the different bacterial groups remained unchanged. For caecal inoculum, caecal contents of 10 slaughter pigs (ca. 105 kg BW) with known origin and feeding (commercial diet without any antimicrobial growth-promoting additive) were sampled and freeze dried. A batch was made of these contents, that was suspended in a sterile 9 g/L NaCl-solution for use in caecal simulations (6.2 total anaerobic bacteria CFU $\log_{10}$/mL suspension). This method was satisfactory for the preparation of the caecal inoculum.

Bacterial enumerations and analysis of bacterial metabolites

BACTERIAL ENUMERATIONS

Bacterial counts (viable counts; CFU $\log_{10}$/mL) on starting media (except for caecal simulations) and after incubation were done using the ring-plate technique (Van Der Heyde & Henderickx, 1963). Serial 10-fold dilutions were made from 1 g aliquots of incubated contents, using a sterilized peptone solution (1 g peptone + 0.4 g agar + 8.5 g NaCl in 1 L aqua destillata). Selective media were used for counting the following bacterial groups: total anaerobic bacteria (Reinforced Clostridial Agar, CM0151, Oxoid, Basingstoke, UK + 0.001% hemin), coliform bacteria (Eosin Methylene Blue Agar,

CM0069, Oxoid), streptococci (Slanetz & Bartley Medium, CM0377, Oxoid) and lactobacilli (Rogosa Agar, CM0627, Oxoid + 0.132% acetic acid). In the second study an additional selective medium was used for counting *E. coli* (Tryptone Bile X-glucuronide Medium, CM0945, Oxoid). For caecal incubations, all manipulations were carried out in 90% N_2 + 10% CO_2 atmosphere. The detection limits for the gastric, jejunal and caecal incubations were 1, 2 and 4 CFU $\log_{10}$/mL respectively. When no colonies could be observed on the plates after incubation, an at random number between 0 and the detection limit was assigned in the first study.

ANALYSIS OF BACTERIAL METABOLITES

In the second study samples from starting media and after incubation were taken and the fermentation was stopped with 2% of 6 mol/L H_2SO_4. Samples were stored at -20°C. SCFA and lactic acid were analysed by a GC-method described by Jensen *et al.* (1995). Ammonia was determined according to the method of Conway (1962). In a microdiffusion cell, ammonia is released from the sample by adding K_2CO_3 and diffuses into a H_3BO_3-solution. After diffusion ammonia is quantitatively titrated with 0.01 mol/L HCl.

Experiments and statistical analysis

PRELIMINARY TEST ON ETHANOL IN THE INCUBATION MEDIA AND EXPONENTIAL GROWTH OF BACTERIA

In order to get insight into and to validate the *in vitro* model applied, preliminary experiments were carried out. To evaluate the effect of the addition of absolute ethanol on the fermentation characteristics, a number of gastric (n=9), jejunal (n=10) and caecal (n=5) simulations were performed whereby a positive control, with 2.4 mL/L absolute ethanol in the starting medium was

compared with a negative control (ethanol was replaced by aqua destillata). In a following experiment the effect of the presence of absolute ethanol on the antimicrobial activity of 1000 mg/L *E*-cinnamaldehyde in jejunal simulations (n=4) was evaluated. Therefore *E*-cinnamaldehyde was added either directly to the medium (without absolute ethanol) or as a solution in absolute ethanol (0.421 g/mL). Comparisons were made by a two-paired Student's t-test using the SPSS 11.5 program software (SPSS Inc., Chicago IL, USA).

To further explore the *in vitro* model, thymol at different concentrations (0, 100, 200, 400, 600 and 1000 mg/L) was tested in a jejunal simulation whereby at various time points during the incubation, samples were taken for counting total anaerobic bacteria.

FIRST SERIES OF EXPERIMENTS: SCREENING FOR ANTIMICROBIAL ACTIVITY AGAINST THE PIG GUT FLORA

In a first series of experiments the 7 EO components were screened for their antimicrobial effects against the main components of the pig gut flora by *in vitro* simulations of the gastric, jejunal and caecal fermentation. EO components were tested in a dose range of 0-1000 mg/L (Table 2). In this series of experiments, tea tree EO was also tested but only in gastric and jejunal simulations. Incubations with this EO were carried out in which the final dose of terpinen-4-ol corresponded to an equal dose of terpinen-4-ol when used solely. Incubations were carried out per type of simulation and included each time two positive control treatments and all treatments with the EO components. All incubations were repeated three times which resulted in three independent replicates for each treatment. For each EO component a statistical analysis was performed per bacterial group and per type of simulation by the GLM ANOVA procedure with the fixed effect of treatment (concentration; see Table 2) and replicate as a random factor to account for variation in the inoculum using the SPSS 11.5 program software. Means for effects of treatment (concentration) were compared using Duncan's multiple range test ($P<0.05$).

SECOND SERIES OF EXPERIMENTS: DOSE-RESPONSE STUDY FOR CARVACROL, THYMOL, EUGENOL AND *E*-CINNAMALDEHYDE AND INTERACTION OF COMBINATIONS

In vitro simulations of the gastric and jejunal fermentation were carried out in order to establish dose-response curves for the *in vitro* antimicrobial activity of carvacrol, thymol, eugenol and *E*-cinnamaldehyde (the components with the highest potential out of the first series of experiments) and to evaluate the interaction of combinations. All components and binary combinations per type of simulation were always tested simultaneously in the same incubation including two positive control treatments. There were three replicates. Components were tested individually at 0, 100, 200, 300, 400 and 600 mg/L (Table 2). All possible binary combinations of the 4 EO components were tested in dose combinations of 100 + 100, 100 + 400, 250 + 250 and 400 + 100 mg/L.

The antimicrobial effect of an EO component was expressed as the reduction in viable counts after incubation compared to the control. Dose-response curves for each EO component were fitted to the data points (x, y) using a quadratic function ($y = bx + cx^2$) with GraphPad Prism 4 software (GraphPad Software Inc., San Diego, USA). The quadratic functions and the statistical output data were used to calculate additional parameters. The 'minimum effect concentration' (MEC) of a component was defined as the concentration that equals an effect that is three times the mean square error giving a probability of 99.7% that the effect is different from zero. A bacteriostatic concentration corresponds to the concentration of the EO component whereby a zero increase of CFU $\log_{10}$/mL during incubation was observed. Δ y $\log_{10}$ CFU/mL stands for the concentration that gives a mean reduction of y $\log_{10}$ CFU/mL compared to control.

The isobole method (Berenbaum, 1985 and Berenbaum, 1989) was used to evaluate the effect of the combinations. The method is based on the assumption of zero interactivity (additivity) of components

in a combination. A zero-interactive response for a combination of two agents can be expressed by the following equation :

$$da/Da + db/Db = 1$$

where da and db are known and represent values of concentrations of the two components in the combination. Da and Db are the concentrations of the components that individually would produce the same effect as the combination. On a graph of the dose-response curves of components A and B, a horizontal line intersects these two curves at points corresponding to Da and Db (x-axis coordinates). Da, Db and the combination (da, db) are iso-effective. Therefore, a solution is to find the horizontal iso-effective straight line (giving a y value), which would determine concentrations of A (Da) and B (Db) to satisfy the above equation. This y value represents the value of the expected effect of a non-interactive combination based on the individual dose-response equations. A two-paired Student's t-test was used to compare the experimentally observed effect with the expected effect of the combination. If an experimentally observed effect of the combination is significantly different from the calculated expected effect, interaction can be inferred, if not, this is a zero-interaction. A second statistical test was performed using the interaction index. Therefore the value of the experimentally observed effect of a combination was inserted into the dose-response equations of each individual component as y to calculate the corresponding value of x (horizontal axis for concentration); Da' and Db'. These values of Da' and Db' were inserted into the equation for zero-interaction and this was called the interaction index. An interaction index equal to one corresponds to zero-interaction. The interaction index was analysed using a one-sample Student's t-test with a test value of one and a probability value of 0.05 as the level of statistical significance. Synergism was inferred if the interaction index was significantly lower than one; antagonism if otherwise.

The analysis of bacterial metabolites was carried out in duplicate on the starting media, the control treatments, the treatments 0, 100, 300 and 600 mg/L for the individual components and 100 + 100

and 250 + 250 mg/L for the combinations. For lactic acid, total SCFA and ammonia a statistical analysis was performed by the GLM ANOVA procedure with the fixed effect of treatment and replicate as a random factor using the SPSS 11.5 program software. Means for effects of treatment were compared using Duncan's multiple range test ($P<0.05$).

RESULTS

Preliminary tests on ethanol in the incubation media and exponential growth of bacteria

No significant differences were found between the positive control, with 2.4 mL/L absolute ethanol in the starting medium and the negative control, except for the number of total anaerobic bacteria in caecal simulations ($P<0.01$). The number of total anaerobic bacteria was lowered from 9.74 to 9.31 $\log_{10}$ CFU/mL. Whether *E*-cinnamaldehyde was added either directly to the medium (without absolute ethanol) or as a solution in absolute ethanol, the number of bacteria in jejunal simulations was not affected ($P>0.05$) (data not shown).

A clear exponential increase in number of viable counts after a lag phase for the treatments 0, 100, 200 and 400 mg/L can be seen in Fig. 1. A concentration of 1000 mg/L thymol reduced progressively the number of total anaerobic bacteria.

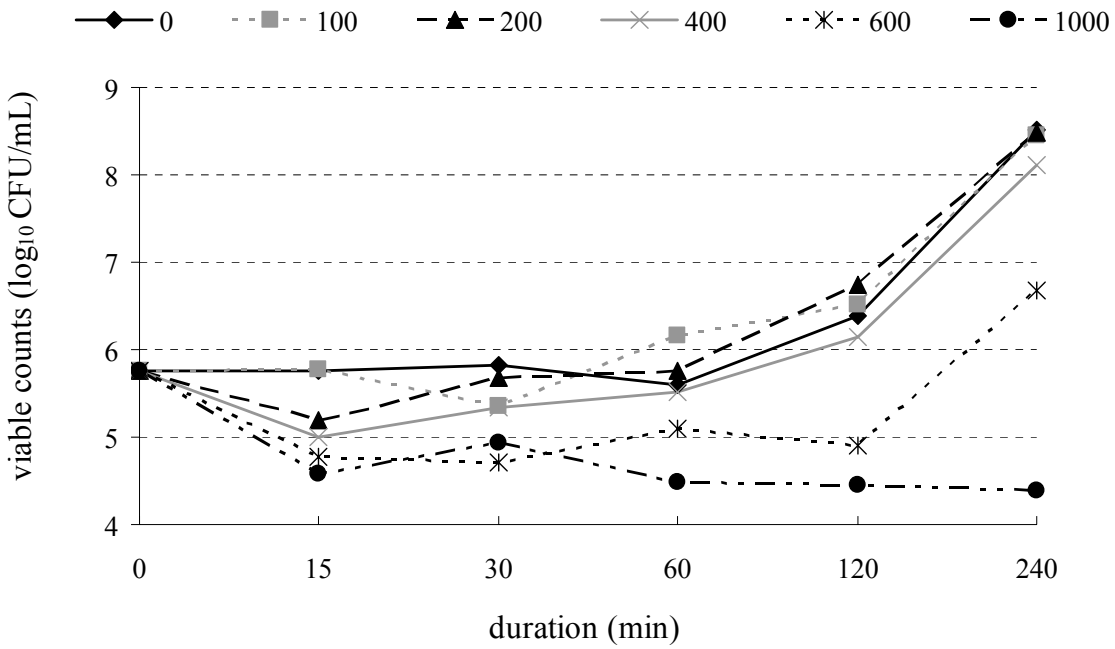


Fig. 1. Effect of thymol at different concentrations (mg/L) on the number of total anaerobic bacteria in a simulation of the jejunal fermentation in a preliminary experiment (n=1)

First series of experiments: screening for antimicrobial activity against the pig gut flora

GASTRIC SIMULATIONS

Thymol had the highest antibacterial activity and showed significantly lower numbers for total anaerobic bacteria and coliforms at 100 and 500 mg/L and for streptococci and lactobacilli at 500 mg/L (Table 3). Carvacrol significantly lowered all bacterial counts at 500 mg/L compared to control, but not at 100 mg/L. *E*-cinnamaldehyde had no effect on streptococci or lactobacilli, but reduced the number of coliforms from 5.83 to 4.54 $\log_{10}$ CFU/mL at 500 mg/L (P<0.05). Eucalyptol, terpinen-4-ol, tea tree EO, *E*-anethole and eugenol had no significant effects (data not shown).

JEJUNAL SIMULATIONS

The number of viable counts in the starting media was relatively low, but this was due to the preservation technique used for the inoculum. The jejunal simulations revealed that carvacrol and thymol had a very strong antimicrobial activity at 1000 mg/L, but no significant differences in bacterial counts compared to the control were found at 100 mg/L (Table 3). *E*-cinnamaldehyde significantly diminished total bacteria and coliform bacteria at 100 and 1000 mg/L. Carvacrol, thymol and *E*-cinnamaldehyde reduced significantly the number of streptococci and lactobacilli at 1000 mg/L; however the reduction by *E*-cinnamaldehyde was clearly smaller than by carvacrol and thymol. Besides the treatment of 100 mg/L (no significant effects), the results for eugenol were comparable to those of *E*-cinnamaldehyde. Only total anaerobic bacteria were significantly reduced by *E*-anethole. Terpinen-4-ol at 1000 mg/L gave a significantly lower number of total anaerobic bacteria (-0.8 $\log_{10}$ CFU/mL) and coliform bacteria (-0.7 $\log_{10}$ CFU/mL). Tea tree EO and eucalyptol showed no significant effects within the dose range tested.

Table 3. Effect of EO components on different bacterial groups (log_{10} CFU/mL) in *in vitro* incubations simulating the pig gut fermentation in the screening experiments (data of treatments with 10 or 20 mg/L not shown) (n=3) (CAR=carvacrol; THY=thymol; ANE=*E*-anethole; EUG=eugenol and CIN=*E*-cinnamaldehyde)

Bacterial group	Concentration (mg/L)	Gastric simulations			Jejunal simulations					Caecal simulations [4]			
		CAR [1]	THY	CIN	CAR	THY	ANE	EUG	CIN	CAR	THY	EUG	CIN
Tot.anaerobic bacteria	*starting media*	*5.09*	*5.09*	*5.09*	*4.57*	*4.57*	*3.91*	*4.57*	*4.57*				
	0	5.78^{a}	5.78^{a}	5.78	6.60^{a}	6.60^{a}	6.22^{a}	6.60^{a}	6.60^{a}	9.39^{a}	9.39^{a}	9.69	9.39^{a}
	100	5.40^{a}	5.11^{b}	5.42	6.42^{a}	5.97^{a}	5.62^{b}	7.05^{a}	5.56^{b}	9.65^{a}	9.34^{a}	9.49	9.47^{a}
	500/1000 [2]	4.18^{b}	4.08^{c}	4.82	3.15^{b}	3.09^{b}	5.51^{b}	4.15^{b}	4.06^{c}	6.79^{b}	7.23^{b}	9.60	6.86^{b}
	RSD [3]	0.50	0.29	0.44	0.56	0.50	0.64	0.51	0.32	0.82	0.71	0.37	0.52
Coliform bacteria	*starting media*	*4.29*	*4.29*	*4.29*	*3.58*	*3.58*	*4.11*	*3.58*	*3.58*				
	0	5.83^{a}	5.83^{a}	5.83^{a}	5.31^{a}	5.31^{a}	4.90	5.31^{a}	5.31^{a}	9.12^{a}	9.12^{a}	9.32^{a}	9.12^{a}
	100	4.76^{a}	4.68^{b}	5.74^{a}	5.23^{a}	4.62^{a}	4.69	5.25^{a}	3.72^{b}	9.67^{a}	9.54^{a}	9.00^{a}	9.32^{a}
	500/1000	2.30^{b}	3.00^{c}	4.54^{b}	1.14^{b}	1.13^{b}	4.30	1.00^{b}	0.81^{c}	6.60^{b}	6.94^{b}	7.49^{b}	6.43^{b}
	RSD	0.97	0.23	0.21	0.71	0.33	0.25	0.83	0.57	0.61	0.62	0.45	0.61
Streptococci	*starting media*	*4.82*	*4.82*	*4.82*	*2.64*	*2.64*	*2.70*	*2.64*	*2.64*				
	0	5.01^{a}	5.01^{a}	5.01	4.36^{a}	4.36^{a}	4.17	4.36^{a}	4.36^{a}	8.80^{a}	8.80^{a}	9.11	8.80^{a}
	100	4.71^{a}	4.77^{a}	4.90	4.17^{a}	4.31^{a}	4.31	4.52^{a}	4.40^{a}	8.61^{a}	8.25^{a}	9.08	8.38^{a}
	500/1000	4.01^{b}	3.60^{b}	4.80	1.52^{b}	1.44^{b}	3.88	3.13^{b}	2.97^{b}	5.30^{b}	6.24^{b}	9.04	6.09^{b}
	RSD	0.35	0.25	0.26	0.43	0.35	0.21	0.16	0.24	0.69	0.72	0.31	0.32

Lactobacilli	*starting media*	*4.87*	*4.87*	*4.87*	*3.81*	*3.81*	*3.83*	*3.81*	*3.81*				
	0	4.74[a]	4.74[a]	4.74	4.63[a]	4.63[a]	4.63	4.63[a]	4.63[a]	8.13[a]	8.13[a]	8.47	8.13[a]
	100	4.49[a]	4.70[a]	4.79	4.54[a]	4.48[a]	4.55	4.49[a]	4.53[a]	7.99[a]	7.82[a]	8.06	7.28[a]
	500/1000	3.93[b]	3.71[b]	4.67	1.56[b]	2.44[b]	4.43	3.84[b]	3.92[b]	4.68[b]	3.68[b]	8.28	4.11[b]
	RSD	0.26	0.32	0.19	0.66	0.36	0.19	0.24	0.27	0.80	0.39	0.45	0.44

(1) Mean values per type of simulation, EO component and bacterial group bearing different superscripts are significantly different at $P<0.05$ (Duncan's multiple range test), (2) 500 mg/L for gastric simulations and 1000 mg/L for jejunal and caecal simulations, (3) RSD: Residual standard deviation, (4) Countings on starting media of caecal simulations were not carried out for all incubations and therefore not included in the table (see also text)

CAECAL SIMULATIONS

Bacterial countings on the starting media were only performed for 2 out of 6 incubations, due to practical reasons and as the same batch inoculum was used for all 6 incubations. The mean number of viable counts in the starting media in the 2 incubations for total anaerobic bacteria, coliforms, streptococci and lactobacilli were respectively 4.02, 2.86, 3.83 and 3.12 $\log_{10}$ CFU/mL. The results for carvacrol, thymol and *E*-cinnamaldehyde were more or less identical (Table 3). The 1000 mg/L treatment gave significant reductions compared to control while the 100 mg/L treatments were not effective. In caecal conditions, eugenol reduced only coliform bacteria. *E*-anethole (7.80 vs. 8.47 $\log_{10}$ CFU/mL) and terpinen-4-ol (7.55 vs. 8.47 $\log_{10}$ CFU/mL) at 1000 mg/L had only a significant effect on lactobacilli. Eucalyptol again was not effective within the dose range tested.

Second series of experiments: dose-response study for carvacrol, thymol, eugenol and E-cinnamaldehyde and interaction of combinations

GASTRIC SIMULATIONS

In gastric simulations the MEC against total anaerobic bacteria for carvacrol and thymol was 159 and 186 mg/L respectively (Table 4). *E*-cinnamaldehyde and eugenol were less effective. The cultivated bacteria were predominantly lactobacilli. This group was reduced with 0.5 $\log_{10}$ CFU/mL by 398 mg/L carvacrol and 433 mg/L thymol, while the effect of *E*-cinnamaldehyde and eugenol at the highest concentration of 600 mg/L was still lower than 0.5. None of the tested combinations resulted in synergism against total anaerobic bacteria or lactobacilli, while some of the combinations showed a trend ($P<0.1$) towards antagonism. Generally, the variability between replicates for gastric simulations was higher than for jejunal simulations which interfered with a smooth analysis of

interaction. The lactic acid concentration ranged between 0.56 and 1.00 mmol/L (control treatment 0.60 mmol/L), without significant differences between treatments.

JEJUNAL SIMULATIONS

The MEC for carvacrol, thymol, eugenol and *E*-cinnamaldehyde against total anaerobic bacteria in jejunal simulations was 255, 258, 223 and 56 mg/L respectively. *E*-cinnamaldehyde showed a strong effect against coliform bacteria (the most abundant group in the jejunal simulations) at low doses (Table 4), however the effect against coliform bacteria at 600 mg/L gave smaller differences between *E*-cinnamaldehyde, carvacrol and thymol (Fig. 2). The activity of the EO components against *E. coli* was similar to their respective activity against coliform bacteria (data not shown). For all groups of bacteria, the estimated parameters of the quadratic function for carvacrol and thymol were not different ($P<0.05$). This was also the case in the gastric simulations.

Few combinations demonstrated synergism; most mixtures showed zero interaction or antagonism. Two combinations of carvacrol + thymol were clearly synergistic against total anaerobic bacteria: 250 mg/L carvacrol + 250 mg/L thymol and 400 mg/L carvacrol + 100 mg/L thymol with an interaction index of 0.91 and 0.94 ($P<0.05$). Following combinations were found to have an antagonistic effect against both coliform bacteria and *E. coli*: 100 mg/L carvacrol + 400 mg/L thymol; 100 mg/L carvacrol + 400 mg/L eugenol; 100 mg/L thymol + 400 mg/L eugenol and 400 mg/L eugenol + 100 mg/L *E*-cinnamaldehyde ($P<0.05$)

Lactic acid concentrations were higher than in the gastric simulations but once again the statistical model did not indicate significant differences between treatments ($P>0.05$) (Table 5). Generally, the treatments with the strongest antimicrobial effects had the lowest concentration of total SCFA in the medium after incubation, with a lower proportion of acetic acid and higher proportions of propionic and butyric acid. Ammonia concentrations after incubation were relatively high (control treatment 2.51 mmol/L). Significant differences were found between treatments, but these were considered not

to be physiologically relevant. Besides, none of the treatments could significantly reduce the concentration of ammonia compared to control.

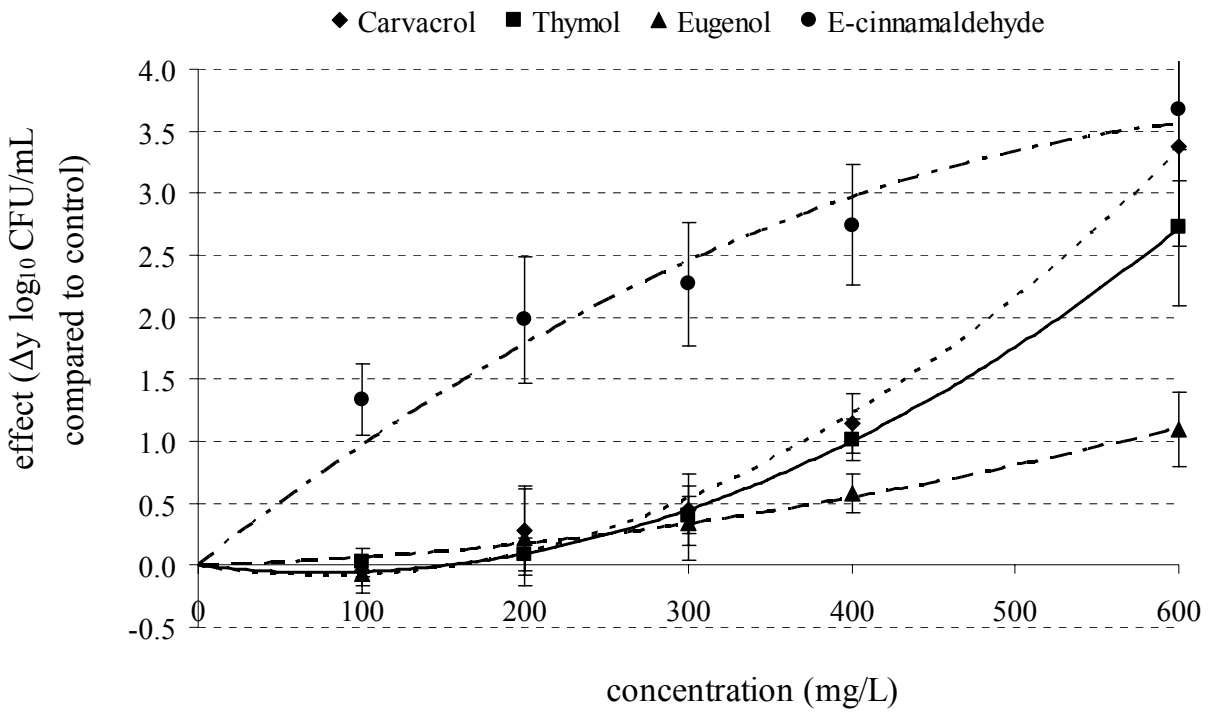


Fig. 2. Quadratic curves fitting data points of effect of EO components on coliform bacteria in jejunal simulations of the pig gut fermentation (n=3) (starting media: 2.77 $\log_{10}$ CFU/mL; control: 5.31 $\log_{10}$ CFU/mL)

Table 4. Data on the antimicrobial activity of carvacrol, thymol, eugenol and *E*-cinnamaldehyde in *in vitro* simulations of the pig gut fermentation in the dose-response study

Bacterial group	Carvacrol	Thymol	Eugenol	*Trans*-cinnamald.
Gastric simulations				
Total anaerobic bacteria (4.54±0.54/4.22±0.29) [(1)]				
MEC [(2)]	159	186	360	302
Δ 0.5 $\log_{10}$ CFU/mL [(3)]	178 ± 76.5	181 ± 84.0	262 ± 102.7	321 ± 133.0
Bacteriostatic conc. [(4)]	-	-	-	-
R^2 [(5)]	0.19	0.21	0.13	0.18
Lactobacilli (4.00±0.65/3.78±0.65)				
MEC	103	117	229	83
Δ 0.5 $\log_{10}$ CFU/mL	398 ± 90.0	433 ± 105.0	> 600	> 600
Bacteriostatic conc.	-	-	-	-
R^2	0.45	0.41	0.30	0.24
Jejunal simulations				
Total anaerobic bacteria (5.01±0.72/6.05±0.51)				
MEC	255	258	223	56
Δ 1 $\log_{10}$ CFU/mL	447 ± 56.4	445 ± 57.4	> 600	225 ± 28.2
Bacteriostatic conc.	457 ± 58.1	455 ± 59.1	> 600	241 ± 30.1
R^2	0.87	0.86	0.63	0.43
Coliform bacteria (2.77±1.02/5.31±0.84)				
MEC	286	323	207	96
Δ 1 $\log_{10}$ CFU/mL	371 ± 61.6	400 ± 74.6	565 ± 68.4	104 ± 44.4
Bacteriostatic conc.	535 ± 100.7	583 ± 125.9	> 600	315 ± 77.4
R^2	0.92	0.86	0.73	0.68
Streptococci (3.05±0.64/4.68±0.33)				
MEC	279	261	399	274
Δ 1 $\log_{10}$ CFU/mL	487 ± 67.2	478 ± 46.0	> 600	483 ± 79.0
Bacteriostatic conc.	> 600	580 ± 61.5	> 600	> 600
R^2	0.79	0.91	0.63	0.68
Lactobacilli (4.21±0.13/4.41±0.13)				
MEC	232	154	496	176
Δ 0.5 $\log_{10}$ CFU/mL	555 ± 55.5	516 ± 40.0	> 600	> 600
Bacteriostatic conc.	300 ± 24.0	275 ± 17.2	556 ± 55.0	385 ± 25.2
R^2	0.43	0.58	0.22	0.40

[(1)] Viable counts ($\log_{10}$ CFU/mL) of starting media and control treatment (n=3), [(2)] MEC, Minimum Effect Concentration: concentration (mg/L) that equals an effect that is three times the mean square error giving a probability of 99.7% that the effect is different from zero, [(3)] Concentration (mg/L) that gives a mean reduction of y $\log_{10}$ CFU/mL compared to control ± standard deviation, [(4)] Concentration (mg/L) whereby a zero increase of viable counts ($\log_{10}$ CFU/mL) during incubation was observed ± standard deviation, [(5)] R^2 of regression analysis using a quadratic function

Table 5. Effect of EO components on the production of bacterial metabolites in jejunal simulations of the pig gut fermentation (CAR=carvacrol; THY=thymol; EUG=eugenol and CIN=*E*-cinnamaldehyde) (data 100 mg/L treatments and 100 + 100 mg/L combinations not shown) (n=3)

Component or combination	Concentrat. (mg/L)	Lactic acid (mmol/L) [1]	SCFA			
			Total (mmol/L)	Molar % acetic acid	Molar % propionic acid	Molar % butyric acid
Start. media	-	*1.26*	*0.29*	*88.7*	*5.8*	*6.5*
Control	0	1.31	0.51^{cd}	89.7	4.0	6.3
CAR	300	1.21	0.48^{bcd}	89.1	4.0	7.0
	600	1.20	0.33^{ab}	84.0	5.5	10.6
THY	300	1.21	0.43^{abcd}	88.6	4.4	7.0
	600	1.35	0.32^{a}	84.6	5.3	10.1
EUG	300	1.24	0.45^{abcd}	88.2	4.5	7.3
	600	1.43	0.38^{abc}	85.3	4.5	10.1
CIN	300	1.22	0.33^{ab}	82.5	7.2	10.3
	600	1.19	0.32^{a}	84.2	5.6	10.2
CAR+THY	250+250	1.31	0.33^{ab}	85.2	6.0	8.7
CAR+EUG	250+250	1.17	0.31^{a}	84.6	5.5	9.9
CAR+CIN	250+250	1.15	0.32^{a}	86.6	5.3	8.1
THY+EUG	250+250	1.17	0.36^{ab}	86.7	5.3	7.9
THY+CIN	250+250	1.31	0.32^{a}	84.1	6.6	9.3
EUG+CIN	250+250	1.26	0.32^{a}	81.9	7.0	11.1
RSD [2]		0.19	0.10			

[1] GLM ANOVA was carried out for lactic acid and total SCFA, mean values per column bearing different superscripts are significantly different at $P<0.05$ (Duncan's multiple range test), [2] RSD: Residual standard deviation.

DISCUSSION

Validation of the in vitro incubation model

The objective of this study was to characterise the antimicrobial activity against the pig gut flora of several promising EO components in an *in vitro* batch incubation model. In Fig. 1 it can be seen that for the control treatment in a jejunal simulation there is an initial lag phase followed by an exponential increase of the number of viable counts of total anaerobic bacteria. In the log growth phase the bacterial cells are highly metabolically active and are particularly sensitive to adverse conditions. In this way the model applied is well suited to evaluate the effect of antimicrobial agents. All EO components used show low water solubility but are well soluble in organic solvents. Therefore a solution of the respective component was made in absolute ethanol and added to the incubation medium and vigorously mixed. The final concentration of ethanol in the incubation medium never exceeded 2.4 mL/L. The addition of these small amounts of ethanol in the incubation medium had no or hardly any effect on the number of bacteria counted after incubation, even if 1000 mg/L *E*-cinnamaldehyde was present. This shows that the addition of absolute ethanol does not affect the fermentation in this assay. The positive control treatment was therefore used as the control treatment in further incubations.

It was concluded that the *in vitro* incubation model was satisfactory to evaluate the antimicrobial properties of the EO components. However, the microbial ecology of the pig gut is very diverse and complex. In our incubations the different bacterial groups considered did not show an equal rate of growth. In the starting media of the gastric and jejunal simulations (Table 3 and 4) the lactobacilli represented the most abundant group. After incubation they were outnumbered by the coliform bacteria. Coliform bacteria have shorter generation times and grow well in rich media like the incubation medium used. Surprisingly in the gastric simulations of the screening study, the number of lactobacilli did not increase while the number of coliform bacteria rose from 4.29 to 5.83 $\log_{10}$

CFU/mL. It has to be stressed that after addition of the artificial substrate and the inoculum to the 0.2 mmol/L glycine buffer (pH 3) the pH of this medium rose up to 3.6 - 4.0 which further increased slightly during incubation. This explains why growth of coliform bacteria could occur. However in the gastric simulations of the dose-response study, no viable counts of coliform bacteria were detected neither in the starting nor after incubation.

The antimicrobial properties of the isomers carvacrol and thymol

Of the 4 tested monoterpenes (carvacrol, thymol, eucalyptol and terpinen-4-ol) only carvacrol and thymol showed interesting antimicrobial effects within the dose-range tested on the flora of the pig gut. Carvacrol and thymol are isomeric chemicals with a phenolic functional moiety. In the screening study carvacrol and thymol showed comparable antimicrobial properties (in all 3 types of simulations) which was confirmed in the dose-response study (equal estimated parameters of the quadratic function). They inhibited significantly and drastically the growth of all groups of bacteria at the highest dose in the first study (500 and 1000 mg/L for gastric and jejunal simulations respectively) (Table 3). For example, in jejunal simulations carvacrol and thymol at 1000 mg/L reduced the number of total anaerobic bacteria from 6.60 to 3.15 and 3.09 $\log_{10}$ CFU/mL respectively, which means a more than 3000-fold reduction.

Two major conclusions can be drawn from the MEC and Δ y $\log_{10}$ CFU/mL values against all bacterial groups in jejunal simulations for both components (Table 4). Carvacrol and thymol display an important activity against all groups of bacteria equally and can therefore be considered as non-selective, which is in line with the results of Cosentino *et al.* (1999), Griffin *et al.* (1999) and Van Zyl *et al.* (2006). However, Si *et al.* (2006b) found differences in sensitivity among a number of pig intestinal bacteria towards both carvacrol and thymol. On the other hand, these values illustrate the fact that these components have only obvious effects on the antimicrobial growth from a certain (critical) concentration on. This is clearly demonstrated by their highly concave quadratic curves for

the effect against coliform bacteria (Fig. 2). The estimated second order coefficients of the quadratic functions for carvacrol and thymol in this Fig. are $1.26\ 10^{-5}$ and $1.01\ 10^{-5}$ respectively. This important characteristic of the phenolic compounds carvacrol and thymol has also been described elsewhere (Juven *et al.*, 1994). It looks like a minimum dose of carvacrol or thymol at the start is needed to inhibit bacterial metabolic activity in an sufficient way, which subsequently hampers bacterial reproduction and becomes bactericidal. Sub-bactericidal doses did however increase the lag phase (Fig. 1). Falcone *et al.* (2007) demonstrated that thymol was active on each phase of the growth cycle of 7 different hygiene-indicating and pathogenic bacteria.

From Table 4 it can also be deduced that the concentration of carvacrol and thymol to reduce lactobacilli with 0.5 $\log_{10}$ CFU/mL was 28 and 16% higher in jejunal than in gastric incubations and that the MEC against total anaerobic bacteria and lactobacilli was lower in gastric incubations than in jejunal incubations. Their activity against the gut flora was therefore higher in gastric than in jejunal simulations. At low pH these molecules would be more undissociated and more hydrophobic, leading to a better binding to the hydrophobic areas of proteins and to a higher affinity for the lipid phase of prokaryotic membranes and enhancing membrane-associated events (Juven *et al.*, 1994 and Sikkema *et al.*, 1995).

In caecal simulations, the 1000 mg/L treatments of carvacrol and thymol gave significant reductions compared to the control for all groups of bacteria, 100 mg/L was however not effective. No intermediate concentrations were tested here. Piva *et al.* (2002) found a 24% reduction of *Enterobacteriaceae* (4.1 vs 5.4 $\log_{10}$ CFU/mL) after 24 h of an *in vitro* caecal fermentation with 400 mg/L carvacrol and Si *et al.* (2006b) found that both thymol (200 mg/L) and carvacrol (300 mg/L) reduced the number of coliforms, but only thymol had an effect on the number of lactobacilli in a 6 h caecal simulation.

Interestingly, in our study the inhibitory effects of carvacrol and thymol on the production of total SCFA were clearly related to their antimicrobial effects against the coliform bacteria in jejunal simulations (Table 5). A shift to higher proportions of propionic and butyric acid was also observed.

The lactic acid concentration was not effected, which is quite surprising taking into account the effects against the lactic acid forming lactobacilli and streptococci.

Minor antimicrobial effects by terpinen-4-ol, eucalyptol and E-anethole

In contrast to other literature data we observed no clear antimicrobial activity of terpinen-4-ol (within the dose range tested). Terpinen-4-ol is a major component of the EO of tea tree (*Melaleuca alternifolia*). The tea tree EO is well recognized for its antiseptic characteristics. In natural sources of terpinen-4-ol, (+)-terpinen-4-ol is the predominant form (65%) (Leach *et al.*, 1993), which was similar to the batch of tea tree EO used in this study. Therefore it was suspected that the different ratio between the enantiomers (1:1 for terpinen-4-ol) or synergistic effects between various components of the tea tree EO could interfere with the antimicrobial properties. However, the antimicrobial activity of tea tree EO here was equal or weaker than that of terpinen-4-ol, which has also been reported by Cox *et al.* (2001). Furthermore from the screening study it was concluded that eucalyptol (no significant effects) and the phenylpropene *E*-anethole (significant effect for total anaerobic bacteria in jejunal simulations and lactobacilli in caecal simulations at 1000 mg/L) did not give interesting and consistent effects.

Selective antimicrobial activity of eugenol and E-cinnamaldehyde

In our study the ranking of antimicrobial activity against both coliform bacteria and *E. coli* in jejunal simulations was *E*-cinnamaldehyde >> carvacrol > thymol > eugenol (Table 3 and 4). This is (partially) different from the results of Friedman *et al.* (2002) who found the following order in a microplate assay based on bactericidal activity 50%: carvacrol > cinnamaldehyde > thymol > eugenol. In the study of Helander *et al.* (1998), *E. coli* O157:H7 in a dilution assay, was nearly equally inhibited by thymol, carvacrol and cinnamaldehyde (MIC; respectively 450, 450 and 396

mg/L). It is therefore surprising that in our *in vitro* simulations *E*-cinnamaldehyde was a much more powerful antimicrobial against both coliform bacteria and *E. coli* than the other components. However, it has to be stressed that the dose effect of these agents against both coliform bacteria and *E. coli* differed among the agents. At low doses (< 400 mg/L) the effect of *E*-cinnamaldehyde exceeds the effect of the phenolic compounds carvacrol and thymol, while the effect at higher doses (600 mg/L) was comparable (different shapes of the quadratic function; Fig. 2). The quadratic function for *E*-cinnamaldehyde represents a convex curve, a progressive increase in the antibacterial effect takes place. Although our concept of MEC is not fully consistent with the more used concept of MIC, the MEC for *E*-cinnamaldehyde against *E. coli* (56 mg/L) (data not shown) was much lower than the MIC values against *E. coli* in the studies of Didry *et al.* (1993) (250 mg/L); Helander *et al.* (1998) (396 mg/L); Kamel (2001) (1000 mg/L); Olasupo *et al.* (2003) (615 mg/L); Kim *et al.* (2004) (250 mg/L against *E. coli* strains O157:H7 and O26 or 500 mg/L against strains ATCC11105 and O111) and Dal Sasso *et al.* (2006) (175 mg/L against *E. coli* strain ATCC or 125-245 mg/L against two other clinical isolates). There is no clear understanding for this strong activity against both coliform bacteria and *E. coli* at low doses in our studies.

However, *E*-cinnamaldehyde at 100 mg/L had no effect on coliform bacteria in gastric simulations (Table 3) while it affected significantly the coliform bacteria in jejunal simulations (3.72 vs. 5.31 $\log_{10}$ CFU/mL for control). Consequently *E*-cinnamaldehyde possesses a lower antimicrobial activity against the coliform bacteria at a lower pH. Possibly in more acidic and aerobic conditions this chemical with an *α,β*-unsaturated aldehyde structure is less active or more prone to oxidation, although synthetic antioxidants were present in the medium. Partial thermal oxidative decomposition of pure cinnamaldehyde (> 60°C) has been demonstrated by Friedman *et al.* (2000). Another remarkable feature is the lack of activity of eugenol in gastric conditions (highest dose 500 mg/L) while it had significant effects at 1000 mg/L against all bacterial groups in jejunal simulations and against coliform bacteria in caecal simulations. Si *et al.* (2006b) found that the antimicrobial activity of clove (containing eugenol) against two strains of *E. coli* and *S.* Typhymurium DT104 was

moderately reduced after a pre-treatment of eugenol at pH 2. These authors questioned the stability of eugenol at low pH. Generally, in our incubations, eugenol had a weaker effect on the pig gut flora than carvacrol, thymol and *E*-cinnamaldehyde.

In contrast to carvacrol and thymol, *E*-cinnamaldehyde showed a clear selective antimicrobial spectrum. It was found to be highly inhibitory for coliform bacteria and *E. coli* while it hardly inhibited the growth of lactobacilli. The effect on streptococci was intermediate. In jejunal simulations a 10-fold reduction of coliform bacteria was achieved with a dose of 104 mg/L vs. 483 mg/L for streptococci. More than 600 mg/L was needed to have an 0.5 $\log_{10}$ CFU/mL effect on lactobacilli. This explains also the higher MEC against total anaerobic bacteria in gastric than in jejunal simulations for *E*-cinnamaldehyde, as the lactobacilli were the predominant group after incubation and that no CFU of coliform bacteria were found on the EMB agar plates. Lee & Ahn (1998) also found that the growth-inhibiting activity of cinnamaldehyde was more pronounced in human intestinal *Clostridium perfringens* and *Bacteroides fragilis*, as compared to the bifidobacteria and *Lactobacillus acidophilus*. Lee *et al.* (2001) reported similar results, however they found clear differences in susceptibility of 3 different *Bifidobacterium* species. Cinnamaldehyde had no bactericidal effect on *Lactobacillus sakei* up to 66000 mg/L over a 1-h incubation (Gill & Holley, 2004). The use of *E*-cinnamaldehyde in the jejunum (for example 100 mg/L) can therefore result in a shift in the microbial ecology in favour of lactic acid producing bacteria and reducing the number of (pathogenic) coliform bacteria.

Little scope to improve the antimicrobial efficacy by making mixtures

Synergism of antimicrobial agents could result in lower total doses needed and costs to obtain the same antimicrobial effect as the individual agents. In particular, effective combinations of these EO components could lower the risk for feed intake reduction caused by their pungent aromas and for residues in the meat. To analyse interactions (zero-interaction, antagonism or synergism) between

components, different approaches are available. The isobole method was chosen because this method is independent of the type of dose-response relation (linear, quadratic …) and the mode of action of the agents in the combination. A major drawback of this method is the fact that the analysis is based on the individual dose-response curves, which should be determined properly. As stated elsewhere, some important variation in data has occurred. This rendered the analysis for interaction a more difficult task.

The combinations 250 mg/L carvacrol + 250 mg/L thymol and 400 mg/L carvacrol + 100 mg/L thymol were found to be synergistic against total anaerobic bacteria in jejunal simulations. The total dose of these combinations is 500 mg/L and this was between 5 and 11% lower than the calculated dose needed by the individual components to have the same effect, which indicates that the synergistic effect was rather small. Using a Fractional Inhibitory Concentration Index for the evaluation of the antimicrobial effect against respiratory pathogens of mixtures, Didry *et al.* (1993) found also that only the combination of carvacrol + thymol out of all binary mixtures of cinnamaldehyde, carvacrol and thymol showed synergism. Zhou *et al.* (2007) reported that several binary combinations of these EO components showed substantial synergistic effects against *S.* Typhymurium. In a study of Lambert *et al.* (2001) carvacrol and thymol were additive. In general, interaction effects differ and vary between experiments and reports. To conclude, there seems to be little scope here to improve the antimicrobial efficacy of these EO components by making mixtures.

CONCLUSION

On the basis of the *in vitro* results presented in this paper, it can be concluded that the EO components carvacrol, thymol, *E*-cinnamaldehyde and to a lesser extent eugenol, have potential (in several ways) to modulate the flora and fermentation pattern in the GIT of pigs. Further research on the use of these components should focus on their kinetic behaviour in the gut and their potential for tolerance/resistance development. Therefore, reliable analytical methods should be developed to quantify these chemicals in feed and digesta.

ACKNOWLEDGEMENTS

Part of this work was financially supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT), Brussels, Belgium. The advices and suggestions from L. Decombel and P. Gerard are sincerely appreciated. The authors are also grateful to A. Ovyn, C. Vermander, A. Maes and E. Turtelboom for skilful technical support.

CHAPTER 3

IN VITRO DEGRADATION AND *IN VIVO* PASSAGE KINETICS OF CARVACROL, THYMOL, EUGENOL AND *E*-CINNAMALDEHYDE ALONG THE GASTROINTESTINAL TRACT OF PIGLETS

Redrafted after:

Michiels, J., Missotten, J., Dierick, N., Fremaut, D., Maene, P. & De Smet, S. (2008). *In vitro* degradation and *in vivo* passage kinetics of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde along the gastrointestinal tract of piglets. Journal of the Science of Food and Agriculture, **88** (13), 2371-2381.

CHAPTER 3

IN VITRO DEGRADATION AND *IN VIVO* PASSAGE KINETICS OF CARVACROL, THYMOL, EUGENOL AND *E*-CINNAMALDEHYDE ALONG THE GASTROINTESTINAL TRACT OF PIGLETS

ABSTRACT

The EO carvacrol, thymol, eugenol and *E*-cinnamaldehyde have well-documented antimicrobial properties and offer therefore an alternative for the antimicrobial growth-promoters in pig feeds. The aim of this work was to determine the degradation and kinetics of these EO along the GIT of piglets which is necessary information for a correct application in pig feeds. None of these compounds was significantly degraded in *in vitro* simulations of the pig gastric fermentation. Carvacrol and thymol were not degraded in jejunal simulations, but significant losses up to 29% were found in caecal simulations. Eugenol and *E*-cinnamaldehyde showed a more pronounced degradation in jejunal and caecal simulations. A single dose mixed with feed (13.0, 13.2, 12.5 and 12.7 mg/kg body weight for carvacrol, thymol, eugenol and *E*-cinnamaldehyde respectively) was given orally to piglets. Half-lives in total digestive tract ranged between 1.84 and 2.05 h, whereby *E*-cinnamaldehyde showed the fastest disappearance. All of these EO were mainly and nearly completely absorbed in the stomach and the proximal SI. Plasma concentrations (sum of free and conjugated compound) peaked at 1.39, 1.35 and 0.83 h for carvacrol, thymol and eugenol respectively and this was accompanied by high concentrations in urine. The four compounds were *in vitro* poorly degraded in the proximal segments

of the GIT of piglets, but degradation is expected in more distal segments. *In vivo*, the EO were mainly and nearly completely absorbed in the stomach and the proximal SI.

KEYWORDS: carvacrol, thymol, eugenol, *E*-cinnamaldehyde, degradation, absorption, kinetics, pigs

INTRODUCTION

The use of antibiotics as AMGP in pig feeds has been banned in the EU from January 2006 (regulation EC/1831/2003) and is under debate in other parts of the world. For decades the use of these AMGP in intensive pig production permitted higher growth rates and better feed conversion ratios in a nearly consistent way (Cromwell, 2002). Their mode of action is not fully understood but there is evidence that they work in part by decreasing the overall numbers and/or specific bacterial species or their metabolic activities in the GIT (Anderson *et al.*, 1999; Corpet, 2000; Gaskins *et al.*, 2002; Visek, 1978). Moreover, in the EU antimicrobial inclusion levels of copper in piglet feed is foreseen to be reduced in the future. In this respect, EO with well documented antimicrobial properties like carvacrol, thymol, eugenol and *E*-cinnamaldehyde have gained renewed interest for use in animal feed.

In previous work we were able to demonstrate in *in vitro* simulations of the pig gut fermentation that the minimum concentration of carvacrol, thymol, eugenol and *E*-cinnamaldehyde to reduce the number of total anaerobic bacteria compared to control with a probability of 99.7% was 255, 258, 223 and 56 mg/L respectively (Michiels *et al.*, 2009 – Chapter 2). Carvacrol and thymol were very effective against lactobacilli, streptococci and coliform bacteria while *E*-cinnamaldehyde inhibited mainly the growth of coliform bacteria. In caecal incubations carvacrol, thymol, eugenol and cinnamon oil showed a high efficacy against *S.* Typhimurium DT104, *E. coli* O157:H7 and *E. coli* K88 with little inhibition towards pig endogenous lactobacilli and bifidobacteria (Si *et al.*, 2006). Therefore, these EO offer an alternative to maintain gut health and performance of young animals. However, no data are yet available about the stability and kinetics of these EO in the GIT of the pig when administered orally. This information is necessary for a correct application and dosage of these compounds in pig feeds and also with regard to potential resistance/tolerance development of gut bacteria against these EO.

This work focuses on the degradation and passage kinetics of these EO in the GIT of the piglet. First an accurate method to quantify these EO was developed. Secondly, *in vitro* incubations simulating the fermentation in different segments of the GIT were performed. Samples taken at the start and at the end of these incubations were analysed for their EO concentration. This allowed estimating the degradation of these compounds in the *in vitro* medium. Finally, a single dose of each of these EO mixed with feed was given to piglets. Following euthanasia at different times post-prandial, digesta and body fluids were sampled to obtain kinetic data.

MATERIALS AND METHODS

Chemicals

Carvacrol (282197), thymol (T0501) and eugenol (E5,179-1) were obtained from Sigma-Aldrich nv/sa (Bornem, Belgium) and *E*-cinnamaldehyde (96320) was provided by Fluka (Bornem, Belgium). Ethyl ethanoate (Sigma-Aldrich; 154857) and 2-isopropylphenol (Fluka; 59720) were used in the GC-method to quantify the EO. The enzymes sulfatase (from *Helix pomatia*, Type H-1, EC 3.1.6.1; Sigma-Aldrich; S9626) and β-glucuronidase (from bovine liver, Type B-1, EC 3.2.1.121; Sigma-Aldrich; G0251) were used for the hydrolysis of phase-II metabolites in plasma, bile and urine samples.

Analytical method to quantify carvacrol, thymol, eugenol and E-cinnamaldehyde in feed and distinct biological matrices

EXTRACTION

All extractions were performed in duplicate. The sample size for extraction was approximately 0.5 g (feed), 1.25 g (digesta from stomach, SI and caecum and faeces) or 1 mL (samples from other matrices: *in vitro* gastric, jejunal and caecal incubation media and pre-treated plasma, bile and urine). The extraction step was carried out in 15 mL vials, made out of glass and sealed with a cap with septum. Two mL aqua destillata was added. Thereafter, each sample was spiked with 50 µL absolute ethanol and 100 µL of a 2-isopropylphenol internal standard solution. The concentration of the internal standard solution was 2.5 mg 2-isopropylphenol per mL ethyl ethanoate for all matrices, except for pre-treated plasma and bile, for which it was 0.1 mg 2-isopropylphenol per mL ethyl ethanoate. Four mL ethyl ethanoate was added as extraction solvent. The extraction was performed by vigorous shaking for 30 s on a vortex apparatus followed by 30 min agitation on an orbital shaker. The samples were then left to equilibrate for 8 to 12 h followed by centrifugation (2000 *g*, 6 min) prior to transfer of the less dense ethyl ethanoate layer. Samples were extracted a second time with 4 mL ethyl ethanoate. The combined ethyl ethanoate extracts were reduced to dryness with nitrogen at room temperature. The residue was redissolved in ethyl ethanoate (500 µL for all matrices except 100 µL for pre-treated plasma and bile). Aliquots (1 µL) were used for GC-analysis.

GC-ANALYSIS

GC was performed using a capillary column 30 m x 0.25 mm ID x 0.25 µm film thickness fused silica. The phase composition was 2,3-di-O-methyl-6-O-*tert*-butyl dimethyl silyl beta cyclodextrin doped into 14% cyanopropylphenyl / 86% dimethyl polysiloxane (Restek Rt-βDEXsm; chiral

separation column; Bellefonte, USA). The average helium carrier gas flow was set to a constant velocity of 40 cm/s, equivalent to 1.90 mL/min. The split ratio of the column was 60:1. The injector temperature was set at 250°C. The column oven temperature started at 110°C for 2 min, then programmed to 170°C at 4°C per min and then to 230°C at 40°C per min, and maintained at 230°C for 4 min. The detector type was a FID, with a temperature of 280°C.

VALIDATION

The analytical method has been validated for linearity, specificity, repeatability and recovery of extraction/drying for each EO and matrix. The following criteria were evaluated (European Commission, 2002): [1] The method should give a linear peak response in the concentration range 5 to 1000 mg/kg or mg/L for the matrices feed, *in vitro* incubation media, digesta, faeces and pre-treated urine and in the range 0.25 to 500 mg/L for pre-treated plasma and bile ($R^2 > 0.99$). For each matrix, 6 samples without EO (blank samples) were prepared. These samples were acidified to pH <2 with 2% of 6 mol/L H_2SO_4 and then spiked with 50 µL of the EO diluted in absolute ethanol giving 6 different final concentrations within the respective dose range. Subsequently, each sample was analysed as described in the previous sections (extraction and GC-analysis) in sixfold. The ratios peak area of EO / peak area of internal standard were calculated and used as dependent variable in linear regression analysis. In each series of analyses of unknown samples, numerous blank samples spiked with the EO were included to determine the response factors necessary for calculation of EO concentration in the unknown samples. [2] No peak signal interference should be observed (specificity). Therefore the signal-noise ratio of all peaks in the area 'retention time of analyte ± 2.5%' must be lower than 3. For each matrix, 10 chromatograms obtained from blank samples were analysed. [3] Repeatability, expressed as coefficient of variation (CV; residual standard deviation relative to the mean) should be lower than $\%CV_H = 2^{(1-0.5\ \log C)}$ (Horwitz equation, whereby C is the mass fraction, expressed as the power of 10) and has been determined for three concentrations (low,

medium and high) (n=6). [4] The percentage of recovery for extraction/drying should range between -20 and +10%. Therefore the ratio peak area of EO / peak area of internal standard of a blank sample spiked with the EO before extraction/drying was compared to the ratio peak area of EO / peak area of internal standard of the respective EO added to an equal concentration after extraction/drying of a blank sample. Recovery has been measured for three concentrations (low, medium and high) (n=4). [5] The detection capability (CCβ), the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (= 5%), has been determined as well.

Degradation of EO in in vitro simulations of the gut fermentation

In order to study the degradation of these EO in the GIT, *in vitro* simulations of the fermentation in the GIT were carried out as described by Michiels *et al.* (2009) (Chapter 2). Briefly, incubations of gastric, jejunal and caecal contents with carvacrol, thymol, eugenol and *E*-cinnamaldehyde were performed. The *in vitro* incubation medium was composed of an artificial substrate, a buffer, a suspension of pig gut bacteria and a solution of the EO in absolute ethanol giving different final concentrations: 0, 10 and 100 mg/L for all three types of simulation, 500 mg/L (gastric simulations) and 1000 mg/L (jejunal and caecal simulations). Incubation parameters closely resembled conditions prevailing *in vivo*. All incubations were carried in glass vessels and in triplicate. In Michiels *et al.*, (2009) (Chapter 2) the effects of the EO against the gut bacteria in these *in vitro* simulations are described. In the current paper, samples from these incubations were taken to determine the degradation. Therefore, from each incubation vessel, 5 mL samples were taken at the start and at the end of the incubation and acidified to pH <2 with 2% of 6 mol/L H_2SO_4 to stop fermentation prior to storage at -20°C until analysis. Aliqouts (1 mL) were taken for quantification of EO as described above. The recovery (the concentration of EO after incubation relative to the concentration at the start of incubation; %) was calculated as a measure for degradation. The mean recovery of three

replicates was compared with a test value of 100% by means of an one-sample Student's t-test (SPSS 11.5 program software; SPSS Inc., Chicago IL, USA).

In vivo absorption and passage kinetics of EO

EXPERIMENTAL DESIGN

Twelve piglets (19.8 ± 0.8 kg BW at the day of sampling, ((Landrace x Large White x Seghers synthetic line) x Piétrain) were individually housed and adapted to a synthetic diet (diet A in Table 1) containing 500 mg of each EO (carvacrol, thymol, eugenol and *E*-cinnamaldehyde) per kg feed over a period of 7 days. The feed was prepared by carefully mixing the EO in a fraction of the corn starch and this was further mixed with the other feed ingredients. Piglets were fed the dry meal twice a day, at 8 a.m. and 5 p.m. for a total of 900-1000 g (approximately 85% of *ad libitum* intake). Water was available *ad libitum* throughout the whole experimental period. The day before sampling, the animals were deprived of their evening meal. At 8 a.m. of the day of sampling (t = 0 h), all animals, except one pig (unfed), were given 300 g of the experimental feed (diet B in Table 1). Diet B was similarly prepared as diet A but contained 1000 mg/kg of each EO (intented dose), and in addition 10 g/kg celite545 coarse and 1 g/kg $C_{36}H_{74}$. Animals were allowed to eat for 20 min. In 4 animals, the intake was lower than the given amount of 300 g. Residual feed and feed spoilage were carefully measured per animal. A corrected feed intake was calculated per pig, giving an average feed intake of 272 ± 25 g (n=11). All piglets were slaughtered at different times post-prandial (t = 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 18 and 24 h). The unfed pig served as control to secure that digesta and body fluids were free of EO and indigestible markers at t = 0 h.

Table 1. Ingredient and analysed nutrient composition of the synthetic diet A (adaptation period) and B (sampling day) in the *in vivo* study (g/kg as fed)

	Diet A	Diet B
Ingredient composition		
Corn starch [(1)]	550.55	537.55
Potato protein [(2)]	215.00	215.00
Sucrose	91.00	91.00
Alphacel [(3)]	50.00	50.00
Soybean oil	35.00	35.00
Pectine NF pure citrus medium [(4)]	17.50	17.50
Monocalcium-phosphate	14.00	14.00
Limestone	13.00	13.00
Sodium chloride	6.50	6.50
L-lysine HCl	1.80	1.80
DL-methionine	0.90	0.90
L-tryptophane	0.25	0.25
Premix Vitamins Pigs 3/2.5 [(5)]	1.30	1.30
Premix Trace elem./min. Pigs ¾ [(5)]	1.20	1.20
Celite545 coarse [(6)]	-	10.00
$C_{36}H_{74}$ [(7)]	-	1.00
Carvacrol	0.50	1.00
Thymol	0.50	1.00
Eugenol	0.50	1.00
E-cinnamaldehyde	0.50	1.00
Analysed nutrient composition		
Dry matter	919	922
Ash	38	49
Crude protein	171	168
Ether extract	40	42
Crude fibre	11	12
4 mol/L HCl insoluble ash	0.40	10.10
$C_{36}H_{74}$	0.00	0.97
Carvacrol	NA (8)	0.947
Thymol	NA	0.958
Eugenol	NA	0.910
E-cinnamaldehyde	NA	0.926

[(1)] Cargill, Ghent, Belgium; [(2)] Avebe b.a., Veendam, the Netherlands; [(3)] MP Biochemicals, LLC 900453, Brussels, Belgium; [(4)] Upjohn, Puurs, Belgium, Item nr. 00 160825 000 5; [(5)] Vitamex N.V., Drongen, Belgium ; [(6)] nr. 22140, Fluka, Bornem, Belgium; [(7)] F-9504, Sigma-Aldrich, Bornem, Belgium ; [(8)] NA, not analysed

SAMPLE PREPARATION

Piglets were electrically stunned and killed by exsanguination. Blood was collected in tubes provided with EDTA as anti-coagulant and was centrifuged at 3000 *g* for 15 min. Thereafter, plasma samples were immediately heat-treated in a water bath at 72°C for 5 min to hamper further enzymatic activity prior to storage at -20°C. An abdominal section was made and samples from the gall- and urinary bladder contents were taken and stored at -20°C. The GIT was removed and partitioned into the following digesta sampling sites: stomach, SI (divided into three segments of similar length and designated as S1, S2 and S3) and caecum. Digesta of each sampling site were quantitatively collected. Aliquots (6 g) of the fresh digesta were taken, acidified to pH <2 with 2% of 6 mol/L H_2SO_4 to stop fermentation and stored at -20°C until analysis. The remaining digesta were frozen, freeze-dried and finally ground to pass a 1 mm screen. Upon analysis, thawed plasma, bile and urine samples were pre-treated to hydrolyze phase-II metabolites. One mL of sample fluid was brought into a 10 mL vial and 0.5 mL β-glucuronidase solution (0.1 g in 50 mL 0.2 mol/L acetate buffer pH 5) and 0.5 mL sulfatase solution (0.25 g in 50 mL cold 0.2% (w/v) NaCl solution, prepared immediately before use) were added. The vial was sealed and incubated overnight in a water bath at 37°C. After incubation, these samples were acidified to pH <2 with 2% of 6 mol/L H_2SO_4.

ANALYSIS

Quantification of EO in feed, fresh digesta and pre-treated plasma, bile and urine samples was performed as described above. The determined EO concentration in pre-treated plasma, bile and urine included the free and conjugated compound. Creatinine in urine was determined according to Yatzidis (1974). Diet A and B and freeze-dried samples from digesta were analysed for 4 mol/L HCl insoluble ash and $C_{36}H_{74}$ (Vulich *et al.*, 1991). Weende components of feeds were determined as specified by Dierick *et al.* (2003).

CALCULATIONS AND STATISTICS

Data on EO in different specimens (piglets) and sampling sites were reported as: [1] Concentration (mg/kg or mg/L); the concentration of the EO in urine is also expressed as the concentration of the EO relative to the concentration of creatinine (mg/g creatinine) in order to account for variation in urine volume. [2] Percentage of intake (%): (concentration of EO in digesta x amount of fresh digesta x 100) / total intake of EO. [3] Recovery (%): (concentration of marker in feed x concentration of EO in digesta x 100) / (concentration of marker in digesta x concentration of EO in feed). Two different indigestible markers were used: 4 mol/L HCl insoluble ash and $C_{36}H_{74}$. Presentations of data as [2] percentage of intake and [3] recovery were only applied for data on EO in digesta of the following sampling sites: stomach, S1, S2, S3 and caecum. The weighted sum of these sampling sites was assigned as total digestive tract. To describe gastric emptying and disappearance from total digestive tract, data (y) were plotted against time (t; h) using a first order model $y = a \cdot e^{-b.t}$; whereby a and b are estimated regression coefficients (Dänicke *et al.*, 2004). The half-life $t_{1/2}$ was calculated as ln2/b. Kinetics in other sampling sites were fitted to the following function using the non-compartmental model $y = a \cdot t^{b} \cdot e^{-c.t}$, whereby t = time and a, b and c are regression coefficients (Dänicke *et al.*, 2004). Regression coefficients were estimated iteratively using the least squares method in GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). The following parameters were calculated from the estimated regression coefficients for the second model: $t_{max} = b/c$ (time at maximum response, h); $y_{max} = a \cdot t_{max}^{b} \cdot e^{-c.t_{max}}$ (maximum response, weight unit or %) and $t_{1/2\ AUC}$ = gammainv (0.5, b + 1, 1 / c) (time which corresponds to the half of the AUC, h). The areas under the time-response curves (AUC) for both models, calculated from t = 0 h to t whereby concentration is < CCβ or to t with the lowest measurable concentration (only for bile and urine), were estimated with the trapezoid rule using the same software program. Total plasma clearance with regard to bioavailability (Cl_T/F) was estimated with the following formula: $Cl_T/F = dose/AUC_{0\rightarrow inf}$.

RESULTS AND DISCUSSION

Analytical method to quantify carvacrol, thymol, eugenol and E-cinnamaldehyde in feed and distinct biological matrices

This work elaborated data on the degradation and passage kinetics of carvacrol, thymol, eugenol and *E*-cinnamaldehyde in the GIT of piglets. A prerequisite is the ability to quantify these compounds in feed and different biological matrices in a reliable and accurate manner. The method that was developed fulfilled all criteria that were specified (linearity, specificity, repeatability and recovery), with the exception of the quantification of *E*-cinnamaldehyde in *in vitro* caecal media, caecal digesta, faeces and pre-treated urine. The first criterion was linearity and this was tested by regression analysis. Intercepts were not significantly different from zero and R^2 values were higher than 0.99 in the range of concentrations applied in this study, with the exception of *E*-cinnamaldehyde in *in vitro* caecal media, caecal digesta, faeces and pre-treated urine. In these 4 matrices, a significant negative intercept was found for *E*-cinnamaldehyde and the R^2 were lower than 0.99. The ratios peak area of EO / peak area of internal standard were also remarkably lower compared to those of *E*-cinnamaldehyde in the other matrices. In none of the cases interfering peaks were observed. All CV for repeatability were below $\%VC_H$ and therefore satisfactory, except for *E*-cinnamaldehyde in pre-treated urine. Excluding *E*-cinnamaldehyde in the 4 matrices mentioned above, the recoveries ranged between 80 and 95%. The CCβ for all EO in *in vitro* incubation media, digesta, faeces and pre-treated urine was in most cases approximately 0.5 mg/kg or mg/L. The CCβ for pre-treated plasma and bile was 0.2 mg/L.

The unsatisfactory results for *E*-cinnamaldehyde in *in vitro* caecal media, caecal digesta, faeces and pre-treated urine points at possible important 'matrix effects'. In these cases significant negative intercepts and recoveries below 50% were found, which indicates that a considerable amount of this

compound is rapidly degraded or adsorption to or interaction with particles of the matrix is taking place once it was added to the acidified blank samples. *E*-cinnamaldehyde contains an oxidizable *α,β*-unsaturated aldehyde function. Biochemical transformation of cinnamaldehyde as described by for example Haarmann & Reimer (2001) or Bock *et al.* (1988) can be excluded here due to the fact that before the addition of *E*-cinnamaldehyde, the blank samples were acidified to pH<2 hampering all enzymatic activity. No hydrolysis is possible for cinnamaldehyde, so it is expected to be stable in the aqueous incubation medium, although it may be slowly oxidized to cinnamic acid (Aromatic Consortium, 2005). Yuan *et al.*, (1992) did not find any oxidation of cinnamaldehyde to cinnamic acid in aqueous solutions. In our study, in some cases peak signals for cinnamic acid were found on the chromatograms but the peak areas were far too low to explain all losses of *E*-cinnamaldehyde. Temperature-dependent and non-enzymatic oxidation and further decomposition to benzaldehyde and glyoxal has been reported by Friedman *et al.* (2000), but this occurred for pure cinnamaldehyde and at temperatures above 60°C. Through reaction with amino groups, cinnamaldehyde can form stable Schiff base intermediates (Yuan *et al.*, 1992) that are insoluble in ethyl ethanoate, the extraction solvent used in our method. Other nucleophilic addition reactions could also be involved. The exact reason for this analytical problem remains unclear and it is also not understood why these problems are found only in these 4 matrices. However, these matrices have in common that they are rich in microbial (*in vitro* caecal medium, caecal digesta and faeces) or animal (pre-treated urine) N-containing degradation products increasing the likelihood for Schiff base formation to occur. It was concluded that the method was not satisfactory to quantify *E*-cinnamaldehyde in these 4 biological matrices.

Degradation of EO in in vitro simulations of the gut fermentation

The EO used are high-boiling liquids or solid in the case of thymol, so it was unlikely that there were any losses due to volatilization during the *in vitro* incubations at 37-39°C or during further handling

at room temperature. So recoveries below 100% are believed to be due to degradation during incubation. The recoveries for gastric incubations were not significantly different from 100% (Table 2), so it is very likely that these EO will not be transformed in the proximal parts of the GIT. Carvacrol and thymol were not degraded in jejunal simulations but significant losses up to 29% were found in caecal simulations (Table 2). The recovery was also considerably higher at the higher dose, which is supported by literature data. At doses higher than in our study (1000-2500 mg/L), Varel (2002) found that carvacrol and thymol were stable in swine waste under anaerobic conditions (gassed with nitrogen) for 62 days, with 90 to 95% of the compound being recovered. Similarly, Broudiscou *et al.* (2007) found no degradation of thymol in an 24 h *in vitro* caprine ruminal fermentation at a dose of 2000 mg/L. However, carvacrol and thymol were found to be biodegraded under aerobic (Chamberlain & Dagley, 1968) and anaerobic conditions (Gibson & Harwood, 2002) mainly by soil bacteria and by a diverse array of pathways. Hence, it is interesting to note that here at the higher doses they are not or little degraded in conditions simulating the pig gut fermentation. Therefore bacterial resistance due to metabolization of these compounds is not anticipated, although long-term exposure to these compounds should be investigated. Indeed, various studies (Ultee *et al.*, 2000; Di Pasqua *et al.*, 2006) highlighted the potential for induced bacterial tolerance in the presence of sub-lethal doses of several EO, which can compromise efficacy when applied *in vivo.*

Significant degradation in jejunal simulations at 100 mg/L was found for both *E*-cinnamaldehyde (27%) and eugenol (8%) and at 1000 mg/L for *E*-cinnamaldehyde (11%) (Table 2). As discussed above, the GC-method could not be validated for quantification of *E*-cinnamaldehyde in the caecal incubation medium. However, there was no detectable response (<CCβ) for *E*-cinnamaldehyde for the 100 mg/L objects after caecal incubation, which could indicate that this compound was (totally) transformed under caecal conditions. The 1000 mg/L objects after incubation gave a moderate response but could neither be quantified. *E*-cinnamaldehyde has shown to be readily biodegradable. It was 100% biodegraded after 21 days in an OECD 301B test (Haarmann & Reimer, 2001). Bock *et al.*

(1988) described the reduction of cinnamaldehyde to the corresponding alcohol by the fungus *Botrytis cinerea*. Microbial degradation of eugenol and cinnamaldehyde involves oxidation of the side chain to carboxylic acid prior to hydroxylation and cleavage of the benzene ring (Shimoni *et al.*, 2002). For example, eugenol is microbially degraded to yield coniferyl alcohol, ferulic acid, vanillin and vanillic acid as intermediates (Tadasa, 1977). Likely, the higher biocatalytic capacity of the caecal bacteria compared to bacteria in the stomach and SI causes degradation of these EO to a larger extent. However, degradation pathways have been described mainly for soil microbes which are not present in the GIT of pigs and pure chemical transformation as discussed above in the case of *E*-cinnamaldehyde, cannot be excluded. Interestingly Friedman *et al.* (2000) found that eugenol protected cinnamaldehyde against heat-induced destruction and Basketter (2000) reviewed the quenching effect of eugenol towards the skin sensitizing activity of cinnamaldehyde in human. In Michiels *et al.* (2009) (Chapter 2) we reported the effects of these EO against the gut bacteria in these *in vitro* simulations. It was shown that eugenol had a clear antimicrobial activity against coliform bacteria in jejunal simulations, while an antimicrobial effect in caecal simulations was not found. Possibly, the partial degradation caused this lack of activity in caecal simulations. *E*-cinnamaldehyde reduced significantly the number of coliform bacteria in jejunal simulations from 5.31 (control) to 3.72 (100 mg/L) and 0.81 $\log_{10}$ CFU/mL (1000 mg/L) and in caecal simulations from 9.12 to 9.32 and 6.43 $\log_{10}$ CFU/mL. It was also found that *E*-cinnamaldehyde had a much stronger effect against coliform bacteria in jejunal simulations as compared to carvacrol and thymol while in caecal simulations these 3 compounds showed a similar inhibition of bacterial growth. The lower activity of *E*-cinnamaldehyde in caecal simulations could be caused by microbial and/or chemical degradation of this compound.

Table 2. Recovery of EO from *in vitro* gastric, jejunal and caecal simulations of the fermentation in the GIT of pigs

	Dose of EO (mg/L)			
	100		500-1000 [1]	
	Recovery (%) [2]	Significance [3]	Recovery (%)	Significance
Gastric simulations				
Carvacrol	91.9	NS	100.6	NS
Thymol	87.5	NS	97.4	NS
Eugenol	91.7	NS	100.1	NS
E-cinnamaldehyde	92.7	NS	88.7	NS
Jejunal simulations				
Carvacrol	106.1	NS	107.9	NS
Thymol	107.3	NS	105.8	NS
Eugenol	92.1	**	99.9	NS
E-cinnamaldehyde	73.2	**	88.9	*
Caecal simulations				
Carvacrol	71.0	*	89.0	NS
Thymol	77.2	*	83.1	*
Eugenol	78.4	*	75.5	*
E-cinnamaldehyde	ND [4]		ND [4]	

[1] 500 mg/L for gastric simulations and 1000 mg/L for jejunal and caecal simulations; [2] recovery = concentration of EO after incubation relative to concentration of EO at the start of incubation, %; [3] NS = not significantly different from 100%, *, ** = significantly different from 100% at 5% and 1% confidence level respectively (n=3); [4] ND, not detectable (see text)

In vivo absorption and passage kinetics of EO

To study the kinetics of carvacrol, thymol, eugenol and *E*-cinnamaldehyde in the GIT of piglets, an experimental layout was chosen wherein animals at the day of sampling were euthanized at different times after a single orally given dose. Each time point was represented by one piglet. Dänicke *et al.* (2004) argued that this seemed to be justified in the view of the regressive treatment of the data and the high experimental and analytical expenditure, which is also the case in this trial. At the day of

sampling, not all the piglets consumed the amount of feed offered. Residual feed and feed spoilage were carefully measured per animal and a corrected feed intake was calculated per piglet, giving an average feed intake of 272 ± 25 g (n=11). The CV for this corrected feed intake was lower than 10% and this was considered acceptable for further analysis. The EO concentration in feed was analytically verified and found to be slightly lower than the intended dose: 946.6, 957.8, 909.8 and 926.4 mg/kg for carvacrol, thymol, eugenol and *E*-cinnamaldehyde respectively (Table 1). An average feed intake of 272 g corresponded therefore with a dose of 13.0, 13.2, 12.5 and 12.7 mg/kg BW for the same EO. All further calculations to determine kinetic data were based on the analytically verified EO concentration in feed. In the week before the day of sampling, animals were adapted to a diet containing the same mixture of EO (intended dose of 500 mg/kg), because preliminary experiments had shown that feed intake in piglets is very low the first day of offering these highly aromatic compounds.

In Table 3 the passage kinetics in stomach and total digestive tract are presented. R^2 were above 0.80, except for *E*-cinnamaldehyde. Half-lives of percentage of intake in total digestive tract ranged between 1.84 and 2.05 h. These values are only slightly higher than the respective values for percentage of intake in stomach, indicating that disappearance from the GIT was running almost parallel to disappearance from stomach. If we assume that the EO are not degraded in the proximal parts of the GIT (see above) disappearance from total digestive tract equals absorption. The half-lives ($t_{1/2}$) and emptying rates (b) for percentage of intake in stomach of all EO were respectively lower and higher than the respective values of dry matter and markers (Table 3). It means that the EO disappeared faster from stomach as compared to dry matter, 4 mol/L HCl insoluble ash (marker for solid phase) and $C_{36}H_{74}$ (marker for lipophilic liquid phase). This might suggest that the EO were absorbed in the stomach and/or emptied with the aqueous phase of the digesta. Since plasma concentrations for carvacrol, thymol and eugenol (sum of free and conjugated compound; *E*-cinnamaldehyde was not retrieved in plasma in none of the specimens) were high in specimen 0.5 h

post-prandial (Fig. 1) and peaked within 1.4 h (Table 4), it can be assumed that absorption occurred in the stomach. Absorption in stomach would depend on solubilisation of the EO. The dry matter content of gastric digesta ranged between 14 and 21% for specimens ≤ 3 h post-prandial. On the other hand, estimated $t_{1/2}$ and emptying rates b for recovery in stomach relative to markers of the EO were respectively higher and lower than the respective values for percentage of intake in stomach (Table 3), which means that part of the EO was not absorbed from stomach. Indeed, stomach emptying coincided with increasing amounts in S1, as can be seen for all compounds in Fig. 1. Nevertheless, the amounts of EO that were recoverd in S1 represented only a minor fraction of the ingested quantity of EO. The AUC for percentage of intake in S1 (Table 4) relative to the AUC for percentage of intake in total digestive tract (Table 3) was 5.7, 6.1, 4.0 and 1.8% for carvacrol, thymol, eugenol and *E*-cinnamaldehyde respectively. Therefore, EO entering the duodenum must be rapidly absorbed. The thorough mixing with pancreatic juice and bile, should lead to solubilisation/emulsification, enhancing their absorption at this section of the SI. This is further substantiated by the prolonged high plasma levels until 4 h post-prandial and the comparable shape of curves for plasma concentration and percentage of intake in S1 (Fig. 1). It is reasonable to state that part of the EO had escaped from solubilisation and absorption in stomach due to adsorption to organic matter. It is well known that these compounds may interact with organic matter. Varel (2002) found that 90 to 95% of the recovered thymol in swine waste was found in the solid fraction. Veldhuizen *et al.* (2007) showed that carvacrol binds to albumin leaving less free unbound carvacrol and cinnamaldehyde reacted easily with proteins (Yuan *et al.*, 1992; Kim *et al.*, 2006). In our experiment the EO were mixed with corn starch before preparing the feed. Hence, a different preparation or formulation (*e.g.* encapsulation) can affect the rate of solubilisation and absorption. Here, *E*-cinnamaldehyde showed the fastest disappearance from total digestive tract (see half-life and emptying rate in Table 3), but it is unlikely that the higher water solubility of *E*-cinnamaldehyde compared to the other 3 compounds can explain this difference.

Small amounts were detected in S2 and S3 (Table 4), for example 1.9% of carvacrol intake (ratio AUC_{S3} to $AUC_{total\ digestive\ tract}$) was found in S3 and the estimated maximum concentration in this sampling site was 9.6 mg/kg digesta. In the digesta of S1, S2 and S3 of the specimens 0.5, 1 and 2 h post-prandial considerable quantities of the markers were recovered (see also t_{max} values for percentage of intake of dry matter and markers in this sampling sites; Table 4). Obviously, gastro-intestinal motility of the 24 h fasted animals caused a rapid shunt of part of the digesta (both solid and liquid phase) to the distal SI. Recovery of the EO in the SI could also be due to enterohepatic re-cycling of free and conjugated compounds. This was certainly not the case for *E*-cinnamaldehyde since this compound was not detected in bile. Carvacrol, thymol and eugenol (sum of free and conjugated compound) were found in bile for specimens t = 0.5 to 6 h and surprisingly for the 24 h specimen. The concentration ranged between 1.40 and 6.93 mg/L and was independent from time post-prandial. Bile secretion is principally continuous but variable and in fasted animals bile can become more concentrated (Sambrook, 1981). An estimation of the contribution of enterohepatic re-cycling was made based on calculated AUC values and an assumed 24 h bile secretion volume of 45 mL/kg BW (Sambrook, 1981). The estimated percentages of intake recovered in bile were 3.2, 4.1 and 2.5% for carvacrol, thymol and eugenol respectively. Probably, this is an overestimation because in specimens euthanized at later times post-prandial bile was more concentrated. The relative amount found in S1 (ratio AUC_{S1} to $AUC_{total\ digestive\ tract}$) for carvacrol, thymol, eugenol and *E*-cinnamaldehyde was 5.7, 6.1, 4.0 and 1.8% respectively. Strikingly, the higher quantities found in S1 for carvacrol, thymol and eugenol as compared with *E*-cinnamaldehyde, the compound that was not detected in bile, nearly equals the estimated contribution of enterohepatic re-cycling. Therefore, enterohepatic re-cycling for carvacrol, thymol and eugenol in piglets as such cannot be neglected, but more detailed studies are necessary to elucidate this phenomenon and its precise contribution to the kinetics in the GIT. It can be concluded that absorption of all EO was fast and occurred mainly and nearly completely in stomach and the proximal SI. This finding has relevance with regard to the formulation of effective inclusion levels in feeds. For example, in this trial with an inclusion level nearly as high

as 1000 mg per kg feed, the concentrations in gastric digesta were reduced by 50% in less than 3 h (Table 3) and none of the estimated maximum concentrations in S1 and S3 were higher than 75 mg/kg and 10 mg/kg respectively (data not shown).

E-cinnamaldehyde has not been detected in any of the plasma samples (< 0.2 mg/L). Yuan *et al.* (1993) retrieved cinnamaldehyde in plasma up to 2.5 mg/L in rat blood after oral gavage of 500 mg/kg BW with a bioavailability of 18% (AUC after oral gavage relative to AUC after *i.v.* injection). At 50 mg/kg BW, a dose almost 4 times higher than in our study, the blood concentrations of cinnamaldehyde were below 0.1 mg/L at all sampling times, which is consistent with our results. It is concluded that absorbed *E*-cinnamaldehyde in piglets is also prone to rapid metabolization leaving undetectable amounts of the parent compound in plasma. The fate of *E*-cinnamaldehyde and its metabolites has not been investigated in this research but literature data indicates that with single dose administration recoveries in urine and faeces always exceeded 80% (for review on metabolic fate of cinnamaldehyde see Bickers *et al.* (2005) and Cocchiara *et al.* (2005)), so residues are not expected. However, 24 h after oral administration of a single dose of ^{14}C-labelled (β-carbon atom) cinnamaldehyde (5-500 mg/kg BW) in rats, between 0.31 and 0.90% and between 0.13 and 0.26% of radioactivity was recovered in fat and muscle respectively (Sapienza *et al.*, 1993).

The calculated Cl_T/F was 15.6, 19.4 and 30.3 L/h for carvacrol, thymol and eugenol respectively. The data represent the clearance for the sum of free and conjugated parent compound with regard to bioavailability. They are much higher than the one found by Kohlert (2001) for thymol in human (1.21 L/h), but comparable or lower than values for other phenolic compounds (phenol, guaiacol and *p*-cresol) in humans (Ogata *et al.*, 1995) or eugenol in rats (Guenette *et al.*, 2007). Our data indicate that clearance of these phenols is fast, which is confirmed by the fact that t_{max} values for concentration in urine are only slightly higher than the ones in plasma (Table 4). A rapid renal excretion occurred since high amounts of the sum of free and conjugated carvacrol, thymol and

eugenol were found in urine (Table 4), which is obviously in agreement with other studies in rodents and humans for thymol and carvacrol (Takada *et al.*, 1979 and Austgulen *et al.*, 1987) and eugenol (Sutton *et al.*, 1985). In these studies also a number of metabolites (*e.g.* oxidation products) have been detected, but for pig no data are available about the metabolic fate of these phenols. Guenette *et al.* (2007) found a $t_{1/2AUC}$ for eugenol in plasma, after repeated oral administrations of 40 mg/kg BW in rats of 14 h which is remarkably higher than the 2.1 h in our study (12.5 mg/kg BW). Probably, species, dose and frequency of administration can greatly influence metabolization and plasma levels. The estimated regression coefficients and kinetic parameters for concentration in plasma given in Table 4, show clearly that metabolization and elimination was faster for eugenol as compared to carvacrol and thymol. Estimated bioavailabilities (F) for these and related compounds range between 16% (underestimated) and 100% (Ogata *et al.*, 1995; Kohlert *et al.*, 2002 and Bhattaram *et al.*, 2002). It is very probable to state that the bioavailabities of carvacrol, thymol and eugenol in this trial must be high because the absorption was fast and nearly complete and large amounts in urine were found. Systemically available thymol (free and conjugated) can contribute to its pharmacological effects observed, for example for treatment of bronchitis and upper respiratory infections (Anonymous, 1996). As Kohlert *et al.* (2002) did not find free thymol in plasma of humans, these authors proposed that cleavage of the sulphate conjugate at the level of alveoli is likely to happen so that free thymol could be effective in the target organ respiratory system. It should be stated that the single dose in that study was very low; 1.08 mg per human volunteer. Few studies reported data on residue analysis in body tissues or pork for these compounds. Zitterl-Eglseer *et al.* (2007) could not detect carvacrol in different tissues, including muscle and abdominal fat and indirectly, a sensory panel was unable to detect a flavour/aroma difference between control and treated (500 mg/kg oregano oleoresin in diet) pork (Janz *et al.*, 2007). However, in both studies the orally administered dose was low compared to high inclusion rates used here and considered necessary when aiming at a modulation of the gut bacterial community (Michiels *et al.*, 2009 – Chapter 2).

Table 3. Passage kinetics of carvacrol, thymol, eugenol and *E*-cinnamaldehyde in the total digestive tract and stomach according to a first order model[(1)] after oral administration of respectively 13.0, 13.2, 12.5 and 12.7 mg/kg BW to piglets

y	Regression coefficient ± SE [(2)]		$t_{1/2}$ (h) [(3)]	AUC [(4)]	R^2	RSD [(5)]
	a	b				
Percentage of intake in total digestive tract (%)						
Carvacrol	61.9 ± 9.4	0.34 ± 0.09	2.05	140	0.87	8.13
Thymol	61.9 ± 9.3	0.34 ± 0.09	2.02	138	0.88	7.91
Eugenol	56.7 ± 9.8	0.35 ± 0.11	1.97	123	0.85	8.21
E-cinnamaldehyde	36.3 ± 9.5	0.38 ± 0.17	1.84	71.9	0.71	7.64
Concentration in stomach (mg/kg)						
Carvacrol	190 ± 18	0.25 ± 0.05	2.74	590	0.93	17.9
Thymol	192 ± 18	0.25 ± 0.04	2.78	604	0.93	18.1
Eugenol	178 ± 23	0.31 ± 0.07	2.27	448	0.90	20.7
E-cinnamaldehyde	130 ± 31	0.34 ± 0.14	2.01	281	0.74	26.2
Percentage of intake in stomach (%)						
Dry matter	71.4 ± 9.5	0.29 ± 0.07	2.38	208	0.88	8.91
4 mol/L HCl insoluble ash	62.5 ± 11.7	0.23 ± 0.08	3.04	203	0.74	12.1
$C_{36}H_{74}$	59.1 ± 12.6	0.25 ± 0.10	2.82	179	0.72	12.6
Carvacrol	58.0 ± 8.8	0.35 ± 0.09	1.99	127	0.88	7.44
Thymol	58.2 ± 8.7	0.35 ± 0.09	1.97	126	0.88	7.27
Eugenol	54.9 ± 9.4	0.36 ± 0.11	1.90	114	0.85	7.74

E-cinnamaldehyde	39.2 ± 10.2	0.38 ± 0.17	1.82	77.0	0.72	8.17
Recovery in stomach rel. to 4 mol/L HCl ins. ash (%)						
Carvacrol	111 ± 12	0.27 ± 0.06	2.54	309	0.91	11.7
Thymol	111 ± 12	0.27 ± 0.05	2.57	312	0.92	11.1
Eugenol	107 ± 16	0.31 ± 0.08	2.22	258	0.87	14.3
E-cinnamaldehyde	73.9 ± 20.1	0.34 ± 0.17	2.06	163	0.69	17.8
Recovery in stomach rel. to $C_{36}H_{74}$ (%)						
Carvacrol	119 ± 13	0.25 ± 0.05	2.78	362	0.90	12.7
Thymol	119 ± 11	0.25 ± 0.05	2.82	364	0.92	11.3
Eugenol	116 ± 16	0.31 ± 0.08	2.26	291	0.88	14.7
E-cinnamaldehyde	78.4 ± 19.9	0.33 ± 0.15	2.08	177	0.72	17.2

(1) regression analysis according to model $y = a \cdot e^{-b.t}$ where t = time (h) and a, b = regression coefficients; (2) SE = standard error of regression coefficient estimation; (3) $t_{1/2}$ = half-life (= ln2/b); (4) area under the curve; (5) RSD = residual standard deviation of the regression

Table 4. Regression analysis of relating the time after a meal containing respectively 13.0, 13.2, 12.5 and 12.7 mg/kg BW of carvacrol, thymol, eugenol and *E*-cinnamaldehyde to the concentration in urine and plasma and percentage of intake in small intestine (S1, S2 and S3) according to a non-compartmental model[(1)] in piglets

y	Regression coefficient ± SE [(2)]			t_{max} [(3)]	y_{max} [(4)]	$t_{1/2AUC}$ (h) [(5)]	AUC [(6)]	R^2	RSD [(7)]
	a	b	c						
Concentration in urine (mg/g creatinine)									
Carvacrol	284E2 ± 177E2	8.00 ± 1.79	3.99 ± 0.86	2.00	2470	2.17	5400	0.93	232
Thymol	359E2 ± 240E2	8.60 ± 2.01	4.30 ± 0.95	2.00	2550	2.15	5390	0.93	245
Eugenol	135E3 ± 822E2	8.99 ± 1.45	4.73 ± 0.75	1.90	5370	2.04	9780	0.96	388
E-cinnamaldehyde [(8)]									
Concentr. in plasma (mg/L)									
Carvacrol	5.77 ± 1.38	0.69 ± 0.35	0.49 ± 0.18	1.39	3.65	2.78	17.1	0.88	0.63
Thymol	3.99 ± 0.94	0.50 ± 0.35	0.37 ± 0.17	1.35	2.80	3.18	13.7	0.84	0.56
Eugenol	4.85 ± 1.49	0.45 ± 0.37	0.54 ± 0.23	0.83	2.85	2.10	8.79	0.91	0.44
E-cinnamaldehyde [(8)]									
Percentage of intake in S1 (%)									
Dry matter	21.3 ± 5.1	0.66 ± 0.28	0.69 ± 0.19	0.96	2.66	1.94	39.4	0.95	1.09
4 mol/L HCl insoluble ash [(9)]									
$C_{36}H_{74}$	31.9 ± 18.8	1.42 ± 0.68	1.21 ± 0.50	1.18	9.69	1.74	26.9	0.88	1.58
Carvacrol	6.41 ± 2.45	1.84 ± 0.59	1.07 ± 0.34	1.71	2.74	2.34	8.03	0.89	0.43

Thymol	6.79 ± 2.53	1.89 ± 0.58	1.10 ± 0.33	1.72	2.86	2.34	8.35	0.89	0.43
Eugenol	4.00 ± 1.57	2.07 ± 0.65	1.15 ± 0.35	1.80	1.71	2.39	4.98	0.88	0.27
E-cinnamaldehyde	3.33 ± 1.15	7.51 ±1.31	3.46 ± 0.58	2.17	0.62	2.37	1.30	0.98	0.04
Percentage of intake in S2 (%)									
Dry matter	11.4 ± 0.7	0.47 ± 0.11	0.38 ± 0.05	1.23	7.81	3.01	42.9	0.98	0.48
4 mol/L HCl insoluble ash	24.1 ± 4.8	0.53 ± 0.27	0.47 ± 0.15	1.13	15.1	2.58	60.8	0.90	2.00
$C_{36}H_{74}$	13.7 ± 7.6	0.34 ± 0.83	0.28 ± 0.38	1.24	10.5	3.73	52.7	0.26	5.40
Carvacrol	0.46 ± 0.11	0.62 ± 0.34	0.45 ± 0.17	1.37	0.30	2.87	1.28	0.88	0.05
Thymol	0.36 ± 0.11	1.04 ± 0.51	0.56 ± 0.23	1.85	0.24	3.06	1.03	0.79	0.06
Eugenol	0.33 ± 0.04	0.54 ± 0.18	0.40 ± 0.09	1.33	0.22	3.02	1.00	0.96	0.02
E-cinnamaldehyde [9]									
Percentage of intake in S3 (%)									
Dry matter	14.2 ± 2.9	0.00 ± 0.0	0.17 ± 0.13	0.00	14.2	4.12	78.8	0.79	2.65
4 mol/L HCl insoluble ash	54.2 ± 12.7	1.59 ± 0.45	0.77 ± 0.20	2.06	34.7	2.93	138	0.88	5.30
$C_{36}H_{74}$	35.3 ± 10.8	1.71 ± 0.72	0.68 ± 0.26	2.53	31.2	3.53	141	0.72	7.28
Carvacrol	0.31 ± 0.13	5.32 ± 1.51	1.68 ± 0.44	3.17	0.70	3.57	2.63	0.89	0.09
Thymol	0.28 ± 0.09	1.38 ± 0.73	0.52 ± 0.25	2.66	0.27	3.97	1.38	0.69	0.08
Eugenol	0.32 ± 0.11	6.95 ± 1.30	2.29 ± 0.40	3.04	0.70	3.33	2.00	0.95	0.06
E-cinnamaldehyde	0.07 ± 0.03	4.64 ± 1.86	1.50 ± 0.55	3.10	0.12	3.54	0.40	0.84	0.02

[1] regression analysis according to model 2, $y = a \cdot t^b \cdot e^{-c.t}$ where t = time (h) and a, b and c = regression coefficients; [2] SE = standard error of regression coefficient estimation; [3] $t_{max} = b/c$ (time at maximum response, h); [4] $y_{max} = a \cdot t_{max}^b \cdot e^{-c.t_{max}}$ (maximum response, weight unit or %); [5] $t_{1/2\ AUC}$ = gammainv (0.5, b + 1, 1 / c) (time which corresponds to the half of the AUC, h); [6] area under the curve; [7] RSD = residual standard deviation of the regression; [8] *E*-cinnamaldehyde was not detected (<CCβ) in urine and plasma in any of the specimens; [9] regression not converged.

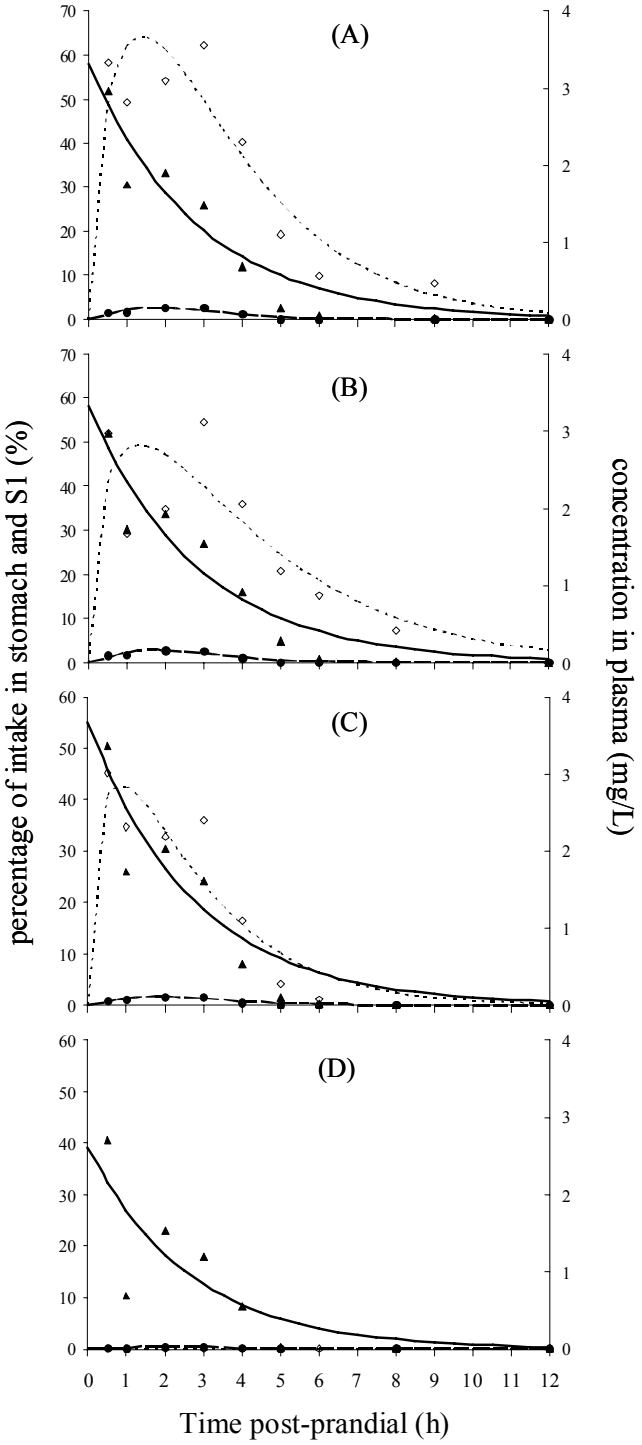


Fig. 1. Percentage of intake in stomach (—▲—) and S1 (—•—; small intestine 1) (left ordinate) and concentration in plasma (-◊-; free and conjugated compound) (right ordinate) for carvacrol (A), thymol (B), eugenol (C) and *E*-cinnamaldehyde (D) in relation to time after oral administration of respectively 13.0, 13.2, 12.5 and 12.7 mg/kg BW to piglets.

CONCLUSION

An analytical method to quantify carvacrol, thymol, eugenol and *E*-cinnamaldehyde in different biological matrices was developed and validated. However, 'matrix' effects rendered the method unreliable for dosing *E*-cinnamaldehyde in *in vitro* caecal media, caecal digesta, faeces and pre-treated urine. These matrices have in common that they are rich in microbial (*in vitro* caecal medium, caecal digesta and faeces) or animal (pre-treated urine) N-containing degradation products increasing the likelihood for Schiff base formation to occur, but this should be further investigated. The four compounds were *in vitro* poorly degraded in the proximal segments of the GIT, but degradation is expected in more distal segments. *In vivo*, the EO were mainly and nearly completely absorbed in the stomach and the proximal SI. The latter finding must be taken into account when designing feed additives based on these EO.

ACKNOWLEDGEMENTS

Part of this work was financially supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT), Brussels, Belgium. The authors are also grateful to A. Ovyn, C. Vermander, A. Maes, E. Turtelboom and Y. De Bock for skilful technical support.

CHAPTER 4

IN VIVO EXPERIMENTS FEEDING CARVACROL AND THYMOL TO PIGLETS

CHAPTER 4A

EFFECT OF DOSE OF THYMOL AND FLAVOURS ON PALATABILITY IN A CHOICE-FEEDING STUDY WITH PIGLETS

CHAPTER 4A

EFFECT OF DOSE OF THYMOL AND FLAVOURS ON PALATABILITY IN A CHOICE-FEEDING STUDY WITH PIGLETS

ABSTRACT

The objectives in this choice-feeding study were: [1] to determine the effect of dose of thymol on palatability (experiment I) and [2] to study the possibility of added flavours to mitigate the aversive effects of high thymol inclusion rates on palatability (experiment II). Twenty-four fully weaned piglets were divided into 8 groups of 3 animals. Each group was housed in a separated pen equipped with two troughs; a reference diet was offered in one through and a test diet in the second trough. Piglets had free access to both troughs and could therefore choose between the two diets offered. Each experiment had two experimental periods that lasted for 4 consecutive days whereby feed intake was recorded daily. Feed intake of the test diet was expressed as the proportion of the total intake and tested by means of a one-sample Student's t-test against a set value of 50% (n=16). The preference of the piglets for feed supplemented with 125, 500 and 2000 mg/kg thymol was 53.0 ± 14.0 % (P>0.05), 47.5 ± 19.6 % (P>0.05) and 9.9 ± 9.2 % (P<0.001) respectively (experiment I). Definitely, 125 and 500 mg/kg thymol did not affect palatability, while the piglets almost completely refused to eat feed with 2000 mg/kg thymol. Two test diets were included in experiment II; feed supplemented with 2000 mg/kg thymol with either flavour A (intended to mask the pungent taste of thymol) or flavour B (designed to mask both taste and smell of thymol) and opposed against a control diet. The relative intake of the test diets was 19.5 ± 10.5 % (P<0.001) and 14.0 ± 15.7 % (P<0.001),

respectively, which means that the flavours could partially alleviate the aversive effects of 2000 mg/kg thymol. Alternatively, animals were offered one of these test diets and a reference diet containing 2000 mg/kg thymol. In accordance to the former results, animals had a preference for the feed with 2000 mg/kg thymol + flavour A (67.6 ± 18.9 %; $P<0.01$). However, the results for the feed with 2000 mg/kg thymol + flavour B (28.9 ± 13.1 %; $P<0.001$) did not confirm the previous observations.

KEYWORDS: thymol, flavour, aroma, palatability, feed intake, pig

INTRODUCTION

The sensorial qualities of a feedstuff are mainly defined by its aroma, texture and flavour (Laitat *et al*., 2003) and determine in part its palatability (reflected by ingestive response). Aroma is appreciated by olfactive (smell) and nasal sensations, while flavour perception can be ascribed to taste and olfactive and thermo-mechanical stimulation like hot or from chemical origin (*e.g.* spicy). The pig's sense of smell is very performant and 2000 times superior to that of human. Generally, pigs accept well acidified feed, are indifferent for salt content, reject feedstuffs with a bitter taste and have a strong preference for sweet substances, in particular sucrose. It has been shown that pigs have preference for specific flavours, and which, when added to feed were able to enchance feed intake in the first weak post-weaning (not in the following weeks) (McLaughlin *et al.,* 1983). However, it is still a matter of debate to what extent flavours can positively influence feed intake.

Flavours are added to feeds to affect the sensorial characteristics of the feed. The objective can be either: [1] to improve the palatability of the feed in order to enhance feed intake, or [2] to mask off-flavours or [3] to standardise the flavour of the feed, because the amount and type of feedstuffs included is changing over time. EO have a distinctive, marked flavour and are traditionally used in feeds as flavour or aroma (*e.g.* Laitat *et al.,* 2004 and list of Sensory additives – flavouring compounds in Community Register of Feed Additives pursuant to Regulation (EC) No 1831/2003; accessed Dec. 2008, http://ec.europa.eu/food/food/animalnutrition/feedadditives - Notified and authorised feed additives). Windisch *et al.* (2007) stated that choice-feeding studies are to be compiled to test palatability. Indeed, a higher feed intake in growth trials can reflect the higher consumption capacity of animals grown larger due to better nutrient utilization compared with untreated controls. In a choice-feeding study, animals have free access to two or more feeds and their preference for one feed can be determined. It is assumed that, when animals can choose between diets that differ only in flavour, their preferred feed reflects the most palatable feed. The number of

published choice-feeding studies with EO is very limited. A dose-related depression of palatability in pigs fed EO from fennel and caraway has been shown (Schöne *et al.*, 2006); as well as from the herbs thyme and oregano (Jugl-Chizzola *et al.*, 2006).

The objectives in this choice-feeding study were: [1] to determine the effect of dose of thymol on palatability and [2] to study the possibility of added flavours to mitigate the aversive effects of high thymol inclusion rates on palatability. Thymol was selected for this trial, because in a preliminary latin-square experiment we observed that thymol had the least depressive effect on appetite compared to the other EO (unpublished results). In that experiment, feed intake was recorded for 6 h after supplying an experimental feed (2000 mg/kg carvacrol, thymol, eugenol and *E*-cinnamaldehyde or control feed), which was preceded by giving control feed and temporarily fasting. For humans, thymol has the characteristic, agreeable odor of thyme and a bitter, aromatic and sometimes burning sensation mediated by receptor mechanisms in the nose and tongue. Hereby, it was shown that this compounds activate TRPA1 (Lee *et al.*, 2007a) and TRPV3 (Xu *et al.,* 2006). This trial was performed to explore the effects of thymol on appetite (enhanced of decreased palatability) in advance to further *in vivo* trials (see Chapter 4B).

MATERIALS AND METHODS

Animals and housing

Twenty-four weaned male castrated piglets (24 d; Belgian Landrace x Large White) with an initial weight of 6.91 ± 0.71 kg were divided into 8 groups of 3 animals with a similar average live weight. Each group of 3 animals was housed in a floor slatted pen (1.20 x 1.25m) equipped with two identical troughs. During the first 10 days post-weaning all animals were offered the basal diet *ad libitum* in both troughs. At 10 days post-weaning the experiments were started. Water was provided *ad libitum*

by two nipple drinkers at the opposite side of the troughs. Pens were cleaned daily to avoid fecal contamination of the pen floor. Average live weight at the start of experiment I and II was 8.04 ± 1.31 kg and 13.06 ± 2.36 kg respectively.

Feed

The composition of the basal diet is given in Table 1. The basal diet was given to all animals until start of experiment I. The basal diet was used to prepare the experimental diets as shown in Table 2. For experiment I the following diets were prepared: a basal diet (CO1), and the basal diet supplemented with thymol at 125 mg/kg (THY125), at 500 mg/kg (THY500) and at 2000 mg/kg (THY2000A). Thymol was added to the feed after adsorption on a cellulose carrier (Alphacel non-nutritive bulk; nr. 900453; MP Biochemicals, LLC, Brussels, Belgium). First 100 g of thymol (T0501; Sigma-Aldrich nv/sa, Bornem, Belgium) was dissolved in 250 mL methanol. Thereafter, 100 g of the carrier was added to the mixture and further blended for 1 h. The mixture was dried in a rotavapor apparatus (waterbath, 30°C) followed by exposure to the air until a dry preparation was obtained. Finally, it was ground and passed through a sieve with 500 µm pores. The preparation of thymol on alphacel contained 48.8 % thymol in the final product. Alphacel was then added to the diets in order to obtain equal amounts of the basal diet in all experimental diets. In experiment II, the diets used were: a basal diet (CO2), and the basal diet supplemented with 2000 mg/kg thymol (THY2000B), with 2000 mg/kg thymol and 4000 mg/kg flavour A (THY2000B+FA) and with 2000 mg/kg thymol and 40000 mg/kg flavour B (THY2000B+FB). The same thymol preparation as in experiment I was used. Flavour A (active principles not disclosed) was provided by Scentarom n.v. (Merchtem, Belgium) and was intended to mask the pungent taste of thymol. Dextrose was the carrier for the active ingredients of this flavour. Flavour B (active principles not disclosed; Scentarom n.v., Merchtem, Belgium) was designed to mask both the taste and smell of thymol. Dextrose was the

main carrier for the active ingredients of flavour B. All experimental diets were prepared in the week before starting the respective experiment.

Table 1. Ingredient and analysed and calculated nutrient composition of the basal diet

Ingredient composition (g/kg)	
Corn	665.6
Soybean meal 43%	282.4
Soybean oil	10.32
DL-methionine	1.36
L-lysine HCl	3.63
L-threonine	0.80
L-tryptophane	0.39
Sodium chloride	6.50
Monocalcium-phosphate	12.50
Limestone	14.00
Premix Vitamins Pigs 3/2.5 [(1)]	1.30
Premix Trace elem. Pigs ¾ [(1)]	1.20
Calculated composition as is [(2)]	
Dry matter (g/kg)	878.6
Crude protein (g/kg)	176.7
Ether extract (g/kg)	40.70
NEv97 (3) (MJ/kg)	9.86
dLYS (g/kg)	10.30
dMET+CYS (g/kg)	6.10
dTHR (g/kg)	5.90
dTRY (g/kg)	1.90

[(1)] Vitamex N.V., Drongen, Belgium – see also Table 1, Chapter 4B; [(2)] CVB Table 1998, Centraal Veevoederbureau, Lelystad, The Netherlands; [(3)] Net energy for pigs, CVB 1998

Table 2. Composition of the experimental diets (g/kg)

Experimental diet	Basal diet	Thymol preparation and carrier [1]		Flavour and carrier			
		Thymol preparation	Alphacel	Flavour A	Flavour B	Carrier A	Carrier B
Experiment I							
CO1	995.90		4.10				
THY125	995.90	0.25	3.85				
THY500	995.90	1.00	3.10				
THY2000A	995.90	4.10					
Experiment II							
CO2	951.90		4.10			4.00	40.00
THY2000B	951.90	4.10				4.00	40.00
THY2000B+FA	951.90	4.10		4.00			40.00
THY2000B+FB	951.90	4.10			40.00	4.00	

[1] The thymol preparation contained 48.8% thymol and alphacel was used as carrier

Choice-feedings experiments

Two choice-feedings experiments were conducted. As a rule, a reference diet was offered in one trough and a test diet was offered in the second trough. Piglets had free access to both troughs and could therefore choose between the two diets offered. Experiment I was started at day 10 post-weaning and tested the palatability of feed supplemented with increasing concentrations of thymol. Four treatments (test diet vs. reference diet) were included: CO1 vs. CO1, THY125 vs. CO1, THY500 vs. CO1 and THY2000A vs. CO1. Each treatment was assigned to two pens in both periods (Table 3). Each experimental period lasted for 4 consecutive days and was preceded by 3 days offering the CO1 diet to all pens in both troughs. At 7.30 a.m. the weighed portions of both diets were offered and 24 h later the residues from both the troughs were weighed. Immediately thereafter, the new portions were offered. Regarding local preferences for the left or right trough, the diets in both troughs were changed every day. The 24-h intake of the test diet was related to the total feed intake. Data from the 4 days were used for statistical analysis which means that there was no

adaptation period to the offered experimental diets. Feed troughs were designed to minimize feed moistening; throughout the whole study moistening was at minimum and not taken into account in further data processing. In case of contamination with urine or feces, feed intake for the respective pen and day was withdrawn from the dataset. Feed intake (kg per day per animal) given in the results section refers to total fresh feed intake. In experiment I each treatment was tested in 4 out of 8 pens for a total of 16 days.

In experiment II the hypothesis was tested whether flavours had the ability to alleviate the depressive effect on palatability of high doses of thymol. Therefore flavours were added to feeds containing 2000 mg/kg thymol. Treatments (test diet vs. reference diet) were: THY2000B+FA vs. CO2, THY2000B+FB vs. CO2, THY2000B+FA vs. THY2000B and THY2000B+FB vs. THY2000B. Similarly to experiment II, each treatment was assigned to two pens in both periods (Table 3). Further protocol details are identical to experiment I.

Table 3. Study design with four treatments (test diet vs. reference diet) allocated to two pens per period in each choice-feeding experiment [(1)]

Pen	Experiment I		Experiment II	
	Period 1 (1)	Period 2	Period 3	Period 4
1	THY500/CO1	CO1/CO1	THY2000B+FA/THY2000B	THY2000B+FB/CO2
2	THY125 /CO1	THY2000A/CO1	THY2000B+FB/CO2	THY2000B+FA/THY2000B
3	CO1/CO1	THY125/CO1	THY2000B+FA/CO2	THY2000B+FB/THY2000B
4	THY2000A/CO1	THY500/CO1	THY2000B+FB/THY2000B	THY2000B+FA/CO2
5	THY2000A/CO1	THY500/CO1	THY2000B+FB/THY2000B	THY2000B+FA/CO2
6	THY500/CO1	CO1/CO1	THY2000B+FA/THY2000B	THY2000B+FB/CO2
7	CO1/CO1	THY125/CO1	THY2000B+FA/CO2	THY2000B+FB/THY2000B
8	THY125/CO1	THY2000A/CO1	THY2000B+FB/CO2	THY2000B+FA/THY2000B

[(1)] Each experimental period lasted for 4 consecutive days and was preceded by 3 days offering the respective control diet to all animals.

Statistical methods

Total feed intake per pen and day were recorded. The intake of the test diet was expressed as the proportion of the total feed intake. Data were analysed for normal distribution using the Kolmogorov-Smirnov procedure in the SPSS 15.0 program (SPSS Inc., Chicago IL, USA). Data of experiment I were analysed by the GLM procedure. Three factors were included in the model: period (2) and day (4) as random factors and treatment (4) as fixed factor. In case a factor was not significant (P>0.05) it was deleted from the model. Treatments means were compared by the Tukey test. If animals have no preference for one of the two diets offered, it can be expected that the relative intake of the test diet equals 50%. Therefore the null hypothesis was that neither of the diets would be preferred and this was tested by means of a one-sample Student's t-test (set value = 50%). The same hypothesis was put forward for analysis of data of experiment II. In this experiment two treatments had the CO2 diet as reference diet. Mean values for relative feed intake of the test diets of these two treatments were compared by a two-sample Student's t-test. The same approach was used for the two treatments whereby THY2000B can be regarded as reference diet.

RESULTS AND DISCUSSION

In experiment I, animals had no preference for one of the two CO1 diets in the treatment CO1 vs. CO1 (Table 4). This means that feed intake from both troughs was equal which is a prerequisite in a choice-feeding study. Further, variance analysis showed that the effects of day and period for relative intake of test diet were not significant. Significant interaction of these factors with the factor treatment was lacking. Therefore it is reasonable to state that there was no learning or habituation to the applied thymol or influence of the preceding treatment. In this study, thymol was prepared on alphacel as an inert carrier. Hereby, the pungent flavour of thymol was preserved, and hence its effect on palatability could be tested. The supplementation with 125 and 500 mg/kg thymol did not affect

the palatability of the diet as is illustrated by *P*-values > 0.05 in the one-sample the Student's t-test (Table 4). However, animals clearly preferred the CO1 diet to a diet with 2000 mg/kg thymol. The animals almost completely refused to eat feed with 2000 mg/kg thymol. In addition, total feed intake was numerically lower for the treatment THY2000A vs. CO1 compared to the other treatments. The reason for this was that in the first two days of the trial the amount offered of each diet was lower than the total feed intake of that day so that animals were limited in their uptake if they wanted to choose for one diet. Probably, this has led to an overestimation of the relative intake of the test diet in this treatment.

From experiment II it can clearly be observed that the animals preferred the CO2 diet compared to the diets supplemented with 2000 mg/kg thymol and either of the two flavours. The relative intake for THY2000B+FA and THY2000B+FB was respectively 19.5 ± 10.5 and 14.0 ± 15.7 % which was higher than the relative intake of THY2000A in experiment I (9.9 ± 9.2 %; overestimated). So, it can be assumed that the flavours were able to partially reduce the aversive effect of 2000 mg/kg thymol on palatability. However, an age effect cannot be ruled out since the animals in experiment II were two weeks older than in experiment I. It is well known that the susceptibility for flavours declines with age (McLaughlin *et al.,* 1983). The relative intake of 67.6 ± 18.9 % for THY2000B+FA compared to the reference diet THY2000B confirms the masking effect of flavour A. The opposite can be said for flavour B, because the animals ate more from the reference diet than from the test diet in treatment THY2000B+FB vs. THY2000B. It can further be questioned whether differences in dextrose content (THY2000B+FB will have the lowest dextrose content) could have interfered with the differences in palatability of the experimental diets. The relative intake for THY2000A in experiment I was 9.9 ± 9.2 % (overestimated), which means that the animals almost completely refused to eat this feed. From this point, it can be understood that it is difficult for flavours to mask substantially the flavour of thymol because the animals rejected feed with 2000 mg/kg in this experiment. Probably, it would have been more appropriate to test flavour addition to a diet

containing for example 1000 mg/kg thymol. This thymol inclusion rate has not been tested here, but from Table 4 it can be expected that the preference for such a diet must be between 9.9 and 47.5%. At least, it can be stated that flavour A, which was intented to mask only taste was more effective than flavour B (intented to mask both taste and smell) in reducing the aversive effects of 2000 mg/kg thymol.

Table 4. Feed intake and relative intake of test diets in two choice-feeding experiments

Treatment	Total feed intake (g per day per animal) [1] [3]	Intake of test diet [2]	
		relative intake (%) [1] [3]	*P*-value [4]
Experiment I			
CO1/CO1	599^{a} ± 193 (307-987)	53.7^{a} ± 16.4 (26.0-85.3)	0.382
THY125/CO1	570^{ab} ± 128 (340-780)	53.0^{a} ± 14.0 (29.2-87.5)	0.428
THY500/CO1	581^{ab} ± 134 (400-920)	47.5^{a} ± 19.6 (12.0-76.7)	0.616
THY2000A/CO1	487^{b} ± 148 (280-727)	9.9^{b} ± 9.2 (2.1-29.5)	<0.001
Experiment II			
THY2000B+FA/CO2	892 ± 166 (600-1267)	19.5 ± 10.5 (3.4-39.1)	<0.001
THY2000B+FB/CO2	866 ± 299 (520-1380)	14.0 ± 15.7 (0-44.4)	<0.001
THY2000B+FA/THY2000B	876 ± 273 (580-1360)	67.6^{a} ± 18.9 (35.8-96.7)	0.003
THY2000B+FB/THY2000B	931 ± 263 (540-1413)	28.9^{b} ± 13.1 (14.2-55.9)	<0.001

[1] Mean ± standard deviation (min-max), n=16; [2] Tests diets in experiment I are CO1, THY125, THY500 and THY2000A; in experiment II, THY2000B+FA and THY2000B+FB were assigned as test diets; [3] Values with different superscripts in the same experiment and column part represent significant differences, $P<0.05$; [4] The null hypothesis was that the test diet was not preferred and this was tested by means of a one-sample Student's t-test (set value = 50%; n = 16).

CONCLUSION

When piglets had free choice between a control diet and a diet with thymol; they had no preference for thymol at 125 mg/kg, neither for thymol at 500 mg/kg. However; palatability was drastically negatively affected when 2000 mg/kg was included in the diet. Addition of flavours was able to partially overcome the reduced intake; especially a flavour that was aimed to mask the pungent taste of thymol.

ACKNOWLEDGEMENTS

Scentarom n.v. (Merchtem, Belgium) is well rewarded for kindly providing flavour A and B. The authors are also grateful to E. Turtelboom, C. Malfait, M. Laseure and P. Van Parys for skilful technical support.

CHAPTER 4B

EFFECT OF DOSE AND FORMULATION OF CARVACROL AND THYMOL ON GUT BACTERIA AND GUT FUNCTION IN PIGLETS AFTER WEANING

Redrafted after

Michiels, J., Missotten, J. A. M., Van Hoorick, A., Ovyn, A., Fremaut, D., De Smet, S. & Dierick, N. A. (revised version submitted). Effect of dose and formulation of carvacrol and thymol on gut bacteria and gut function in piglets after weaning. Animal.

CHAPTER 4B

EFFECT OF DOSE AND FORMULATION OF CARVACROL AND THYMOL ON GUT BACTERIA AND GUT FUNCTION IN PIGLETS AFTER WEANING

ABSTRACT

Two trials were conducted to study the effect of dose and formulation of carvacrol and thymol on gut bacterial counts and metabolites and gut function in weaned piglets. In experiment I, 25 piglets (28 d, 6.59 ± 0.48 kg) were allocated to five dietary treatments: a control diet and the same diet supplemented with either carvacrol or thymol at doses of 500 and 2000 mg/kg. The EO were dissolved in the soybean oil fraction, prior to mixing with the basal diet. In experiment II, 35 piglets (28 d, 7.99 ± 0.73 kg) were assigned to seven dietary treatments: the same control diet as in experiment I and this diet supplemented with thymol in one of three formulations (on celite, on alphacel or microencapsulated) at doses of 500 and 2000 mg/kg. Microencapsulation was applied in order to delay the release of the active ingredient in the gastro-intestinal tract. All diets were given *ad libitum*. At 11/12 days post-weaning piglets were euthanized, and digesta from stomach, proximal and distal SI were sampled for bacteriological (plating onto selective media for total anaerobic bacteria, coliforms, streptococci and lactobacilli) and biochemical analysis. Small intestinal tissue was sampled for histo-morphological determinations. Feed intake was higher for animals fed 500 mg/kg carvacrol and thymol, with the exception of the microencapsulation treatment (between +12.4 and +44.7% as compared to control), and likewise villi at the distal SI were larger compared to control (between +4.9 and +7.5% as compared to control; $P>0.05$). In experiment I, the villus/crypt

ratio at the distal SI for the experimental diets (1.30-1.32) was higher than for the control diet (1.24) (P<0.05). Thymol fed animals in experiment II had a lower number of intra-epithelial lymphocytes at the proximal (P<0.05) and at the distal (P<0.1) SI as compared to the control animals. Concurrently, the villus/crypt ratio at the distal SI tended to be higher in the experimental diets (P<0.1). Mean EO concentration in stomach and proximal SI for the 2000 mg/kg diets ranged between 475 and 647 and between 5 and 24 mg/kg fresh digesta, respectively. Cumulative absorption in proximal SI was higher than 90% for all treatments. These data suggest that EO can enhance feed intake at 500 mg/kg and improve gut health, but conclusive evidence for clear antimicrobial effects towards the major culturable bacteria of the pig gut is limited.

KEYWORDS: carvacrol, thymol, gut bacteria, gut function, weaning piglets

INTRODUCTION

Pigs post-weaning are subject to growth-check and vulnerable for gastro-intestinal disorders and infections, often leading to diarrhoea and consequently economic losses. In the past decades, the preventive use of antibiotics and metals (copper and zinc) as AMGP has proven its benefits. The AMGP have proven to elicit their effects through modulation or reduction of the bacterial metabolism and load in the gastrointestinal tract of monogastrics, resulting in improved gut functionality and health and animal performance (Decuypere *et al.,* 1991; Visek, 1978; Gaskins *et al.*, 2002). Recent limitations on the use of antibiotics are believed to worsen the challenges for the young pig during the critical period post-weaning. Moreover, in the EU antimicrobial levels of copper are foreseen to be reduced in the near future.

Plant-based products have gained interest as alternative. In a recent review by Windisch *et al.* (2008) it is concluded that reported effects on pig performance gives a rather scattered picture, and that the mode of action of plant-based compounds is not always well understood. Most attention has been directed to compounds with antimicrobial activities, like oregano oil, thyme oil and their components carvacrol and thymol. The antimicrobial properties of carvacrol and thymol have been shown in standard assays (*e.g*. Karapinar and Aktug, 1987; Didry *et al.*, 1993) and against commensal bacteria and pathogens in *in vitro* simulations of the pig gut fermentation (Michiels *et al.*, in press – Chapter 2; Si *et al.*, 2006). Michiels *et al.* (in press) (Chapter 2) showed that carvacrol and thymol equally inhibited the main culturable bacterial groups present. However, they only exerted a bactericidal effect above a certain (critical) concentration. It was found that 400 and 400-550 mg/kg digesta would be necessary to induce a substantial reduction in number of coliforms and lactobacilli, respectively. Si *et al.* (2006) found, in a 6 h caecal incubation, that carvacrol (300 mg/L) and thymol (200 mg/L) inhibited growth of coliforms, but only thymol was effective against lactobacilli. The number of *in vivo* trials with these pure compounds is however limited (Piva *et al.*, 2007b; Trevisi *et*

al., 2007). Up to date there is no solid evidence that these compounds exert an antimicrobial effect *in vivo* towards gut bacteria in pigs. Besides, both compounds have been shown to possess antioxidant properties (*e.g.* Youdim and Deans, 2000), to affect the immune response by intracellular Ca^{2+} mobilization in monocytic cells (Chan *et al.,* 2005), and to inhibit the release of elastase (Braga *et al.,* 2006) and of cyclooxygenase activity (Marsik *et al.* 2005). Further, Michiels *et al.* (2008) demonstrated that carvacrol and thymol were mainly and nearly completely absorbed in the stomach and proximal SI after oral administration of a single dose mixed with feed (13.0 and 13.2 mg/kg BW for carvacrol and thymol) to piglets. Fast absorption is likely to reduce lumen concentrations and consequently the effectiveness of the EO. Hence, the type of formulation of these compounds in feed might affect the outcome. For example, microencapsulation is assumed to protect the active ingredient from absorption, resulting in a more gradual release in the GIT. However, a variety of techniques to encapsulate active ingredients are available and information on the rate and extent of release of microencapsulated active ingredients applied in pig nutrition is still scarce (Piva *et al.*, 1997; Piva *et al.*, 2007a).

The aim of the present study was to test the hypothesis that carvacrol and thymol could reduce the bacterial load and improve indices of gut functionality in weaned piglets. Two dose levels (500 and 2000 mg/kg) were applied, and in addition different formulations types, including microencapsulation, were tested.

MATERIALS AND METHODS

Animals and feed

In experiment I, 25 newly weaned male-castrated piglets (28 d, BW 6.59 ± 0.48 kg, Belgian Landrace x Large White) were allocated according to live weight to five dietary treatments (pens) of five animals each. Feed was offered *ad libitum* as dry meal. Water was available by nipple drinkers. In

experiment II, 35 newly weaned male-castrated piglets (28 d, BW 7.99 ± 0.73 kg; Hypor hybrid x Piétrain) were allocated according to live weight to seven dietary treatments (pens) of five piglets each. The same basal diet based on wheat and peas was used for both trials (Table 1). Celite 545 coarse (22140, Sigma-Aldrich nv/sa, Bornem, Belgium) was included as source of 4 mol/L HCl insoluble ash. Experiment I consisted of a control (CON) diet (=basal diet) and four experimental diets: CON + 500 mg/kg carvacrol (28219-7, Sigma-Aldrich nv/sa) (CAR500), CON + 500 mg/kg thymol (T0501, Sigma-Aldrich nv/sa) (THY500), CON + 2000 mg/kg carvacrol (CAR2000) and CON + 2000 mg/kg thymol (THY2000). The analytically verified concentration of carvacrol or thymol of the feeds was 0 (CON), 506 (CAR500), 494 (THY500), 1883 (CAR2000) and 1870 (THY2000) mg/kg. The EO were dissolved in the soybean oil fraction and then further mixed with the basal diet, without soybean oil. In experiment II, two thymol inclusion rates and three formulation types were tested. The diets were: CON, CON + 500 mg/kg thymol on celite 545 coarse as carrier (CE500), CON + 500 mg/kg thymol on alphacel non-nutritive bulk (nr. 900453, MP Biochemicals, LLC, Brussels, Belgium) as carrier (AL500), CON + 500 mg/kg microencapsulated (SODA Feed Ingredients, Monaco) thymol (ME500), CON + 2000 mg/kg thymol on celite 545 coarse as carrier (CE2000), CON + 2000 mg/kg thymol on alphacel non-nutritive bulk as carrier (AL2000) and CON + 2000 mg/kg microencapsulated (SODA Feed Ingredients, Monaco) thymol (ME2000). The analytically verified concentration of thymol of the diets was 0 (CON), 474 (CE500), 485 (AL500), 340 (ME500), 2164 (CE2000), 2080 (AL2000) and 1783 (ME2000) mg/kg. Experimental diets were prepared by equally diluting the basal diet with the preparation containing thymol and with alphacel non-nutritive bulk if necessary. For the preparation of thymol on a carrier, firstly 100 g of thymol was dissolved in 250 mL methanol. Thereafter, 100 g of the respective carrier was added to the mixture and further blended for 1 h. The mixture was dried in a rotavapor apparatus (water bath, 30°C) followed by exposure to the air until a dry preparation was obtained. This was ground and passed through a sieve with 500 µm pores. Microencapsulation was applied in order to protect the active ingredient from absorption, resulting in a more gradual release in the gastro-intestinal tract (GIT)

(Piva *et al.*, 1997; Piva *et al.*, 2007a). The microencapsulated thymol was manufactured by complex coacervation in which a double coating protects the thymol. The inner coating was a hydrogenated vegetable oil matrix and the outer layer was made from a pig skin gelatine protein. The preparation of thymol on celite 545 coarse, on alphacel non-nutritive bulk and microencapsulated thymol contained 479, 478 and 197 g/kg thymol respectively. The analytical recovery of thymol in the preparations exceeded 90%.

Table 1. Ingredient and analysed and calculated nutrient composition of basal diet used in the two experiments with weaned piglets.

	Experiment I	Experiment II
Ingredient composition (g/kg as fed)		
Pea	120.00	120.00
Wheat	660.24	660.24
Wheat gluten	120.00	120.00
Soybean oil	30.00	30.00
Sucrose	20.00	20.00
DL-Methionine	2.26	2.26
L-Lysine HCl	7.50	7.50
L-Threonine	2.50	2.50
L-Tryptophane	0.50	0.50
L-Valine	1.00	1.00
Sodium chloride	4.50	4.50
Monocalcium-phosphate	9.00	9.00
Limestone	10.00	10.00
Premix Vitamins Pigs 3/2.5 (1)	1.30	1.30
Premix Trace elem. Pigs ¾ (2)	1.20	1.20
Celite 545 coarse	10.00	10.00
Analysed nutrient composition (g/kg as fed)		
Dry matter	898	913
Ash	46	44
Crude protein	206	196
Ether extract	41	53
Crude fibre	27	28
4 mol/L HCl insoluble ash	7.9	9.6
Calculated nutrient composition (3)		
NEv97 (4) (MJ/kg)	10.28	10.28
CP (g/kg)	202.12	202.12
dLYS (g/kg)	10.25	10.25
dMET+CYS (g/kg)	8.48	8.48
dTHR (g/kg)	6.78	6.78
dTRY (g/kg)	2.08	2.08

(1) Vitamex N.V., Drongen, Belgium (Vit A, 17355 IE/kg as fed; Vit D3, 2314 IE/kg; Vit E, 55250 µg/kg; Vit K3, 1888 µg/kg; Vit B1, 1739 µg/kg; Vit B2, 6240 µg/kg; Vit B3, 20768 µg/kg; Vit B6, 3380 µg/kg; Vit B12, 496 µg/kg and antioxidants (BHT and ethoxyquin), 23400 µg/kg); (2) Vitamex N.V., Drongen, Belgium (Fe, 122400 µg/kg as fed; Cu, 10008 µg/kg; Zn, 99960 µg/kg; Mn, 80040 µg/kg; I, 967 µg/kg; Co, 1008 µg/kg and Se, 350 µg/kg); (3) CVB Table (1997), Centraal Veevoederbureau, Lelystad, The Netherlands; (4) Net energy for pigs, CVB (1997)

Experimental design and sampling strategy

The experiments lasted for 12 days in a temperature-controlled facility (27-28°C). Faeces and health condition of the piglets was visually checked daily and coded per pen. Animals were weighed individually at arrival, at day six and before euthanasia. Feed intake was recorded per pen for the periods 0 to 6 days and 7 to 12 days. Fresh faeces were collected from each pen at day 6 and 12. After freeze-drying, excreta were pooled per diet group. In experiment I, 15 and 10 piglets were sacrificed on day 11 and 12 respectively, without previous fasting. In experiment II, 21 and 14 piglets were sacrificed on day 11 and 12 respectively. Piglets were electrically stunned and killed by exsanguination. Blood was collected in tubes provided with EDTA as anti-coagulant for EO analysis. An abdominal section was made and samples from the urinary bladder contents were taken and stored at -20°C. Subsequently the GIT was removed and partitioned into the following digesta sampling sites: stomach, proximal SI (S1, 0 to 3m distal from pylorus) and distal SI (S2, 3 to 0 m proximal to caecum). Digesta of each segment were quantitatively collected and pH was determined. One g of fresh digesta was taken and processed for bacteriological analysis. Aliquots of the fresh digesta were taken, acidified to pH<2 with 2% of 6 mol/L H_2SO_4 to stop fermentation and stored at -20°C pending analysis for EO and bacterial metabolites. The remaining digesta were frozen, freeze-dried and finally ground to pass a 1 mm sieve. From proximal (3m distal to pylorus, J1) and distal (3m proximal to caecum, J2) SI, segments (10 cm of length) were taken, flushed with 0.9% saline and fixed in neutral buffered formalin for 24 h pending further measurements of histo-morphological parameters.

Chemical, bacteriological and histo-morphological analyses

Quantification of the EO in formulations, feed, fresh digesta, plasma and urine samples was performed as described by Michiels *et al.* (2008) (Chapter 3). Briefly, samples were extracted twice

with ethyl ethanoate whereby 2-isopropylphenol was used as internal standard. The combined ethyl ethanoate extracts were reduced to dryness with nitrogen at room temperature and the residue was re-dissolved in ethyl ethanoate. Aliquots (1 µL) were used for GC-analysis. Plasma and urine samples were pre-treated with β-glucuronidase and sulfatase to hydrolyze phase-II metabolites. Thus, the analyzed content of the EO in plasma and urine includes the free and conjugated parent compound. Creatinine in urine was determined according to Yatzidis (1974).

Bacterial counts (viable counts; $\log_{10}$ CFU/g fresh digesta) in digesta of stomach, S1 and S2 were done using the ring-plate technique (Van Der Heyde and Henderickx, 1963). Serial 10-fold dilutions were made from 1 g aliquots of fresh digesta, using a sterilized peptone solution (1 g peptone + 0.4 g agar + 8.5 g NaCl in 1 L aq. dest.) and plated onto selective media in duplicate. Selective media were used for counting the following bacterial groups: total anaerobic bacteria (Reinforced Clostridial Agar, CM0151, Oxoid, Basingstoke, UK + 0.001% hemin; incubated for 48 h at 37°C under 90% N_2 and 10% CO_2), coliform bacteria (Eosin Methylene Blue Agar, CM0069, Oxoid; incubated for 24 h at 37°C aerobically), streptococci (Slanetz & Bartley Medium, CM0377, Oxoid; incubated for 48 h at 37°C aerobically) and lactobacilli (Rogosa Agar, CM0627, Oxoid + 0.132% acetic acid; incubated for 48 h at 37°C under 90% N_2 and 10% CO_2). Bacterial metabolites were determined in digesta of S2. Short chain fatty acids (SCFA) and lactic acid were analysed by a GC method described by Jensen *et al.* (1995) and ammonia was determined as specified by Van Nevel *et al.* (2003). Diet and freeze-dried samples from gastric digesta, pooled (per pen) digesta of S1, pooled (per pen) digesta of S2 and pooled (per pen) excreta were used to quantify 4 mol/L HCl insoluble ash as indigestible marker to calculate apparent digestibility coefficients. This marker was also used to calculate the apparent digestibility (%) of carvacrol and thymol in the stomach and SI by the formula: $100 - (EO_{comp} \times M_{feed}) / (EO_{feed} \times M_{comp}) \times 100$, in which EO_{comp} and M_{comp} are the concentration of respectively the compound and the marker in the respective section of the GIT and EO_{feed} and M_{feed} are the concentration of respectively the compound and the marker in the feed. Since Michiels *et al.* (2008) showed that degradation of both EO in stomach and SI is not anticipated, the calculated

digested fraction represents the amount that is absorbed. Analysis of dry matter, ether extract, nitrogen and crude fibre of diets, pooled digesta of S2 and pooled excreta were determined according to the standard methods as indicated by Van Nevel *et al.* (2003). Measurement of the histo-morphological parameters villus length (V) and crypt depth (C) and enumeration of intra-epithelial lymphocytes (IEL) were carried as described by Van Nevel *et al.* (2003).

Statistical analysis

The effect of diet was tested using the one-way ANOVA procedure. Animal was used as the experimental unit, which was in agreement with other authors (see *e.g.* Manzanilla *et al.*, 2004; Mikkelsen and Jensen, 2004). Orthogonal contrasts were applied in order to explore the effect of treatments. All calculations were carried out using the SPSS 15.0 program for Windows (SPSS Inc.,Chicago IL,USA).

RESULTS

Experiment I

During the whole trial all animals showed a good health condition and severe diarrhoea was not observed. Animals fed CAR2000 showed a lower average daily gain compared to CON (12.6 vs. 75.3 g/day, $P<0.05$). Animals fed CAR500 and THY500 had a higher feed intake on pen level as compared to CON, +12.4 and +21.9% respectively, although growth was not different from CON ($P>0.05$). The digesta from S2 of animals fed THY2000 had a higher dry matter content compared to CON ($P<0.05$, Table 2). Bacteriological counts in the digesta of stomach, S1 and S2 were in none of the cases significantly lower for the experimental diets compared to CON (Table 2). THY2000 had higher numbers of streptococci (S2) ($P<0.05$) and tended to have more lactobacilli (S1 and S2)

(P<0.1) than in CON. The orthogonal contrast 500 mg/kg vs. 2000 mg/kg for streptococci and lactobacilli counts in S2 was significant, whereby the 2000 mg/kg treatments showed the highest numbers of these lactic acid bacteria. Regarding the same bacterial groups in S2, supplementation with carvacrol gave lower counts than with thymol (orthogonal contrast III). At J2, the ratio V/C for the experimental diets (1.30 - 1.32) was higher than for CON (1.24) (orthogonal contrast of all experimental diets vs. CON, P<0.05, data not shown). This altered ratio V/C was due to enlarged villi (+7.5%; for both CAR500 and THY500, P>0.05) or to shortened crypts (-12.5 and -4.4% for CAR2000 and THY2000 respectively, P>0.05). The concentrations of carvacrol and thymol (2000 mg/kg) were on average 521 and 544 mg/kg fresh digesta in stomach and 5 and 16 mg/kg digesta in S1 (Table 3). For stomach, S1, plasma and urine, the concentration of EO was affected by dose level (orthogonal contrast III), but not for S2. More thymol was retrieved in S1 (P<0.05) and in S2 (P<0.1) than carvacrol. Using 4 mol/L HCl insoluble ash as a marker, it was found that between 25 and 39% (no differences between treatments) and between 50 and 66% of the EO had disappeared (absorbed) from gastric and S1 digesta respectively (Figure 1).

Experiment II

Mild diarrhoea was seen in the pens fed the ME diets from day 5 to 10, but health status of all animals was not compromised. No medical interventions were done during the trial. Feed intake on pen level was not reduced by supplementation of 2000 mg/kg thymol, except for the CE2000 treatment (-20.0%). The diets CE500, AL500, ME500 and AL2000 showed respectively a 44.7, 16.7, 0.4 and 18.3% higher feed intake vs. CON (167.4 g/day). For the whole trial none of the experimental diets altered the average daily gain compared to CON (84.2 g/day), although the average daily gain of animals fed CE2000 tended to be reduced (P<0.1). Differences in dry matter weight of gastric (P<0.1), proximal small intestinal (P<0.1) and distal small intestinal (P<0.05) digesta between the 500 mg/kg and the 2000 mg/kg treatments were found (orthogonal contrast II,

Table 4). Diet ME2000 showed a lower dry matter weight of gastric digesta compared to CON (P<0.05), while the same diet and diets ME500 and AL2000 showed more fill of the distal small intestinal segment as compared to CON (P<0.05). ME2000 had a lower gastric pH (P<0.1) than CON, while the pH of digesta in the S2 segment of animals fed CE2000 was higher than CON (P<0.05). In Table 4 bacteriological counts are shown as well. Diet CE500 (total anaerobic bacteria in S1) and diet CE2000 (total anaerobic bacteria in S1, streptococci in S1 and S2 and lactobacilli in S2) showed lower numbers of bacteria in the SI as compared to CON (P<0.05) and also in comparison to the other formulation types (see orthogonal contrast III and IV). The number of coliforms in S2 was higher in the experimental diets vs. CON (P<0.05), especially for CE500, AL500 and AL2000, and higher for the 500 mg/kg vs. 2000 mg/kg diets (P<0.05). Diet ME2000 gave in all three sampling sites the lowest number of coliform bacteria and the highest number of streptococci and in S2 the number of lactobacilli was higher on this diet compared to the other diets; however these effects were non-significant. The lower numbers of lactic acid bacteria in S2 can explain the lower lactate concentration (P<0.05) as observed for treatment CE2000 (Table 4). At J2, the experimental diets tended to have smaller crypts and an increased V/C ratio (orthogonal contrast I, Table 5). In general, experimental diets gave lower number of IEL at J1 (P<0.05) and at J2 (P<0.1) compared to CON. The number of IEL at J1 and J2 for both diets CE500 and ME2000 were significantly lower compared to CON.

The mean thymol concentration in gastric contents for the 2000 mg/kg treatments ranged between 475 and 647 mg/kg (Table 6). Within each dose level, the thymol content in gastric digesta was lower for the ME treatments than for the other formulation types (orthogonal contrast III and IV). This can be explained by the fact that the analytically verified dose of thymol in the ME diets was lower than in the other diets. Conversely, in the SI, there were no significant differences in the thymol concentration within dose. An equal cumulative absorption in the different segments of the GIT for all treatments was observed (Figure 1). Cumulative absorption in S2 was for all treatments above

98.1%. Therefore it is reasonable to state that the type of formulation did not affect the kinetics of EO along the GIT. Thymol concentration (sum of free and conjugated compound) in plasma and urine were comparable to the results of experiment I.

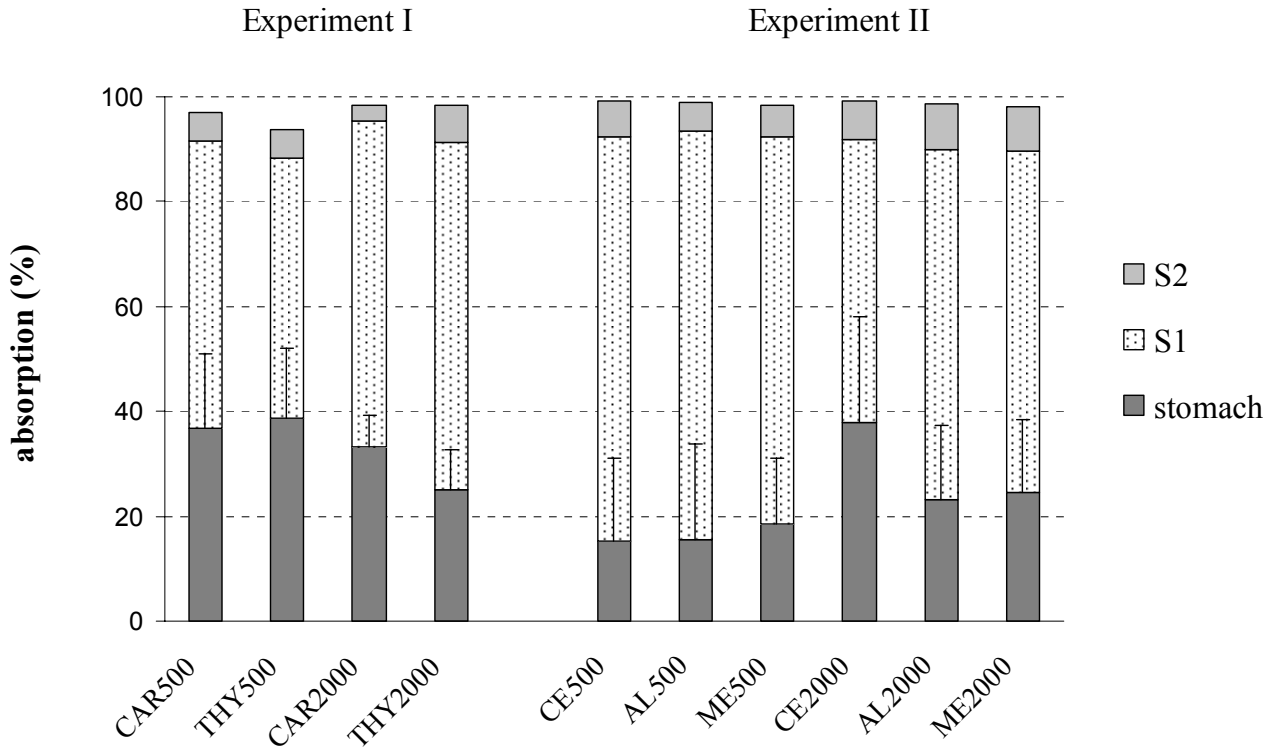


Fig. 1. Cumulative absorption of carvacrol and thymol in stomach, proximal SI (S1) and distal SI (S2) in experiment I and II with weaned piglets.

- Abbreviations are: CAR = carvacrol; THY = thymol; CE = thymol on celite 545 coarse as carrier; AL = thymol on alphacel non-nutritive bulk as carrier; ME = microencapsulated thymol, number indicate dose in mg/kg as fed;

- 4 mol/L HCl insoluble ash was used as indigestible marker; no significant (P>0.05) differences were found between treatments for absorption in stomach in both experiments; determination of absorption in S1 and S2 was based on pooled digesta.

Table 2. Effects of dose (mg/kg) and EO on pH, absolute dry matter weight (g), dry matter content (g/kg), bacteriological counts (log_{10} CFU/g fresh digesta) and bacterial metabolites (µmol/g) of digesta of stomach, proximal (S1) and distal small intestine (S2) in experiment I with weaned piglets.

	CON	500		2000		SE	Significance (1) (2) (3)		
		CAR	THY	CAR	THY		I	II	III
Stomach									
pH	3.5	3.4	3.4	3.5	3.2	0.4	n.s.	n.s.	n.s.
Dry matter	97.0	88.5	77.6	93.9	106.2	17.6	n.s.	n.s.	n.s.
Dry matter content	292.1	308.7	273.0	303.5	288.2	35.2	n.s.	n.s.	n.s.
Total anaerobic bacteria	7.95	8.12	7.85	8.06	7.91	0.22	n.s.	n.s.	*
Coliform bacteria	3.63	4.67	2.95	5.05	4.99	1.30	n.s.	*	n.s.
Streptococci	7.75	8.06	7.81	7.99	8.07	0.36	n.s.	n.s.	n.s.
Lactobacilli	8.49	8.45	8.38	8.39	8.50	0.13	n.s.	n.s.	n.s.
S1									
pH	6.0	6.0	5.8	6.1	6.0	0.2	n.s.	n.s.	n.s.
Dry matter	4.2	5.2	3.4	3.1	4.0	1.4	n.s.	n.s.	n.s.
Dry matter content	88.0	91.5	85.8	78.1	111.4	34.8	n.s.	n.s.	n.s.
Total anaerobic bacteria	6.70	6.73	6.70	6.69	6.84	0.56	n.s.	n.s.	n.s.
Coliform bacteria	2.67	2.42	3.53	2.99	2.57	0.81	n.s.	n.s.	n.s.
Streptococci	6.50	6.70	6.43	5.99	6.83	0.80	n.s.	n.s.	n.s.
Lactobacilli	7.17	7.25	7.32	6.96	7.72(§)	0.43	n.s.	n.s.	*
S2									
pH	6.7	6.7	6.3§	6.3	6.4	0.4	n.s.	n.s.	n.s.

Dry matter	6.6	8.2	4.6	4.7	8.9	3.4	n.s.	n.s.	n.s.
Dry matter content	58.0	93.7	61.7	63.0	113.3§	51.0	n.s.	n.s.	n.s.
Total anaerobic bacteria	7.31	7.23	7.21	7.76	7.57	0.57	n.s.	n.s.	n.s.
Coliform bacteria	3.77	2.93	4.52	4.18	4.13	0.88	n.s.	n.s.	T
Streptococci	7.62	7.29	7.64	7.67	8.42§	0.54	n.s.	*	*
Lactobacilli	8.11	7.71(§)	8.13	8.29	8.52(§)	0.35	n.s.	**	T
Acetate	3.56	4.23	3.31	4.75	4.30	1.53	n.s.	n.s.	n.s.
Propionate	0.28	0.33	0.33	0.30	0.36§	0.12	n.s.	n.s.	n.s.
Butyrate	0.22	0.25	0.24	0.25	0.18	0.04	n.s.	n.s.	T
Lactate	32.8	32.0	50.3	54.9	55.6	24.7	n.s.	n.s.	n.s.
Ammonia	5.57	7.56	5.30	5.94	5.26	3.37	n.s.	n.s.	n.s.

Abbreviations are: CON = control diet ; CAR = carvacrol; THY = thymol and SE = standard error.

(1) n.s. = non-significant (P>0.1); T = trend (P<0.1); * = P<0.05; ** = P<0.01; *** = P<0.001; (2) I = orthogonal contrast of all experimental diets vs. CON; II = orthogonal contrast 500 mg/kg vs. 2000 mg/kg and III = orthogonal contrast carvacrol vs. thymol; (3) orthogonal contrast of each experimental diet vs. CON, mean values of experimental diet with superscript (§) and § are significantly different from CON at respectively P<0.1 (trend) and P<0.05.

Table 3. Effects of dose (mg/kg) and EO on concentration of EO in samples of gastric, proximal small intestinal (S1) and distal small intestinal (S2) digesta, in plasma and in urine in experiment I with weaned piglets (1).

	500		2000		SE	Significance (2) (3)			
	CAR	THY	CAR	THY		I	II	III	IV
Digesta (mg/kg fresh digesta)									
Stomach	118	115	521	544	53.2	n.s.	n.s.	***	n.s.
S1	3.49	3.49	5.02	16.4	6.72	n.s.	*	*	T
S2	2.00	1.64	1.78	5.51	2.87	n.s.	T	n.s.	n.s.
Plasma (µg/mL)	3.10	2.10	10.2	8.76	2.97	n.s.	n.s.	***	n.s.
Urine (µg/mg creatinine)	158	210	665	983	299	n.s.	n.s.	***	n.s.

Abbreviations are: CON = control diet ; CAR = carvacrol; THY = thymol and SE = standard error.

(1) carvacrol and thymol were only found in the sampling sites of the respective diets and were not detected in the control treatment; determined concentration in plasma and urine includes both the free and conjugated parent compound; (2) n.s. = non-significant (P>0.1); T = trend (P<0.1); * = P<0.05; ** = P<0.01; *** = P<0.001; (3) I = orthogonal contrast CAR500 vs. THY500; II = orthogonal contrast CAR2000 vs. THY2000; III = orthogonal contrast 500 mg/kg vs. 2000 mg/kg and IV = orthogonal contrast carvacrol vs. thymol.

Table 4. Effects of dose (mg/kg) of thymol and formulation type on pH, absolute dry matter weight (g), dry matter content (g/kg), bacteriological counts (log_{10} CFU/g fresh digesta) and bacterial metabolites (µmol/g) of digesta of stomach, proximal (S1) and distal small intestine (S2) in experiment II with weaned piglets.

	CON	500			2000			SE	Significance (1) (2) (3)				
		CE	AL	ME	CE	AL	ME		I	II	III	IV	V
Stomach													
pH	3.9	3.8	3.9	3.9	4.0	3.9	3.6(§)	0.3	n.s.	n.s.	n.s.	n.s.	n.s.
Dry matter	123.5	105.2	118.5	103.9	85.9(§)	101.6	69.5§	28.2	n.s.	T	n.s.	n.s.	n.s.
Dry matter content	301.2	285.2	320.4	305.6	301.9	319.0	304.0	30.6	n.s.	n.s.	n.s.	n.s.	n.s.
Total anaerobic bacteria	8.72	8.69	9.09§	8.67	9.00	8.71	8.48	0.29	n.s.	n.s.	n.s.	*	*
Coliform bacteria	4.17	3.92	4.92	3.99	4.76	4.69	3.30	1.28	n.s.	n.s.	n.s.	n.s.	T
Streptococci	7.78	7.88	8.08	7.47	7.38	7.73	8.12	0.43	n.s.	n.s.	n.s.	n.s.	n.s.
Lactobacilli	8.43	8.49	8.57	8.73	8.46	8.56	8.27	0.42	n.s.	n.s.	n.s.	n.s.	n.s.
S1													
pH	5.9	5.8	5.7	5.9	6.0	5.7	5.8	0.2	n.s.	n.s.	n.s.	T	n.s.
Dry matter	4.4	5.8	3.5	2.6	3.4	10.0	5.3	1.8	n.s.	T	n.s.	n.s.	n.s.
Dry matter content	85.2	81.2	97.8	84.3	89.0	128.5	114.6	42.0	n.s.	*	n.s.	n.s.	n.s.
Total anaerobic bacteria	7.86	7.08§	7.70	7.70	7.26§	7.53	7.72	0.45	n.s.	n.s.	*	*	n.s.
Coliform bacteria	4.26	4.69	4.70	5.13	5.03	5.67	3.21	1.76	n.s.	n.s.	n.s.	n.s.	n.s.
Streptococci	6.87	6.45	6.90	6.58	6.07§	6.66	7.20	0.58	n.s.	n.s.	T	*	n.s.
Lactobacilli	7.65	7.49	7.73	7.74	7.62	7.65	7.59	0.65	n.s.	n.s.	n.s.	n.s.	n.s.
S2													
pH	5.9	6.2	6.1	6.2	6.5§	6.0	6.1	0.4	n.s.	n.s.	n.s.	n.s.	n.s.

Dry matter	6.0	8.0	5.8	11.4§	8.3	13.9§	10.8§	3.0	*	*	n.s.	T	n.s.
Dry matter content	125.7	83.1(§)	72.2§	118.0	103.1	129.3	106.2	35.4	n.s.	T	n.s.	n.s.	n.s.
Total anaerobic bacteria	8.99	8.99	9.19	8.92	8.55	9.22	9.08	0.48	n.s.	n.s.	T	n.s.	n.s.
Coliform bacteria	5.21	7.87§	8.27§	7.94	6.76	7.98§	5.16	1.70	*	*	n.s.	n.s.	*
Streptococci	7.96	7.92	8.02	7.40	6.90§	7.56	8.14	0.70	n.s.	n.s.	n.s.	n.s.	n.s.
Lactobacilli	8.86	8.74	9.06	8.62	8.01§	8.98	9.09	0.49	n.s.	n.s.	**	*	n.s.
Acetate	11.2	11.1	12.1	3.60	9.88	18.9	4.85	11.8	n.s.	n.s.	n.s.	n.s.	*
Propionate	0.30	1.03	0.00	0.31	0.00	0.00	0.00	0.94	n.s.	n.s.	n.s.	n.s.	n.s.
Butyrate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
Lactate	89.5	76.1	87.2	69.6	24.6§	90.3	79.0	38.7	n.s.	n.s.	*	n.s.	n.s.
Ammonia	3.49	3.18	3.07	4.25	2.71	3.82	4.25	1.08	n.s.	n.s.	n.s.	*	n.s.

Abbreviations are: CON = control diet ; CE = thymol on celite 545 coarse as carrier; AL = thymol on alphacel non-nutritive bulk as carrier; ME = microencapsulated thymol and SE = standard error.

(1) n.s. = non-significant (P>0.1); T = trend (P<0.1); * = P<0.05; ** = P<0.01; *** = P<0.001; (2) I = orthogonal contrast of all experimental diets vs. CON; II = orthogonal contrast 500 mg/kg vs. 2000 mg/kg; III = orthogonal contrast CE vs. AL; IV = orthogonal contrast CE vs. ME and V = orthogonal contrast AL vs. ME; (3) orthogonal contrast of each experimental diet vs. CON, mean values of experimental diet with superscript (§) and § are significantly different from CON at respectively P<0.1 (trend) and P<0.05.

Table 5. Effects of dose of thymol (mg/kg) and formulation type on histo-morphological parameters at 3m distal to pylorus (J1) and 3m proximal to caecum (J2) in experiment II with weaned piglets.

	CON	500			2000			SE	Significance (1) (2) (3)				
		CE	AL	ME	CE	AL	ME		I	II	III	IV	V
J1													
V (µm)	449.9	454.4	430.3	430.9	425.1	464.1	496.2	58.9	n.s.	n.s.	n.s.	n.s.	n.s.
C (µm)	250.2	245.4	229.2	230.0	229.5	248.5	252.4	27.1	n.s.	n.s.	n.s.	n.s.	n.s.
ΔVC (µm)	700.1	699.8	659.5	660.9	654.6	712.7	748.6	81.2	n.s.	n.s.	n.s.	n.s.	n.s.
V/C	1.84	1.89	1.97	1.93	1.89	1.91	2.01	0.19	n.s.	n.s.	n.s.	n.s.	n.s.
IEL (#/100 enterocytes)	26.8	24.0§	25.8	26.6	26.9	24.9	21.9§	1.9	*	n.s.	n.s.	n.s.	n.s.
J2													
V (µm)	428.8	449.6	451.8	413.6	402.8	421.6	424.0	42.3	n.s.	n.s.	n.s.	n.s.	n.s.
C (µm)	244.3	248.7	222.6	221.8	227.8	220.7(§)	218.2(§)	21.1	T	n.s.	T	T	n.s.
ΔVC (µm)	673.1	698.3	674.4	635.4	630.6	642.3	642.2	57.7	n.s.	n.s.	n.s.	n.s.	n.s.
V/C	1.80	1.85	2.08§	1.91	1.82	2.01(§)	1.99(§)	0.16	T	n.s.	**	n.s.	n.s.
IEL (#/100 enterocytes)	26.2	24.5§	25.1	24.7	26.1	24.8	24.7§	2.6	T	n.s.	n.s.	n.s.	n.s.

Abbreviations are: CON = control diet ; CE = thymol on celite 545 coarse as carrier; AL = thymol on alphacel non-nutritive bulk as carrier; ME = microencapsulated thymol; V = villus height; C = crypt depth; IEL = intra-epithelial lymphocytes and SE = standard error.

(1) n.s. = non-significant (P>0.1); T = trend (P<0.1); * = P<0.05; ** = P<0.01; *** = P<0.001; (2) I = orthogonal contrast of all experimental diets vs. CON; II = orthogonal contrast 500 mg/kg vs. 2000 mg/kg; III = orthogonal contrast CE vs. AL; IV = orthogonal contrast CE vs. ME and V = orthogonal contrast AL vs. ME; (3) orthogonal contrast of each experimental diet vs. CON, mean values of experimental diet with superscript (§) and § are significantly different from CON at respectively P<0.1 (trend) and P<0.05.

Table 6. Effects of dose of thymol (mg/kg) and formulation type on concentration of thymol in samples of gastric, proximal small intestinal (S1) and distal small intestinal (S2) digesta, in plasma and in urine in experiment II with weaned piglets (1).

	500			2000			SE	Significance (2) (3)			
	CE	AL	ME	CE	AL	ME		I	II	III	IV
Digesta (mg/kg fresh digesta)											
Stomach	138	151	105	614	647	475	75.6	***	n.s.	*	**
S1	2.96	3.04	3.33	13.0	23.8	21.3	8.81	***	n.s.	n.s.	n.s.
S2	1.63	1.80	1.12	4.41	4.55	7.47	3.78	**	n.s.	n.s.	n.s.
Plasma (µg/mL)	2.63	2.37	2.42	8.03	7.87	7.91	2.91	***	n.s.	n.s.	n.s.
Urine (µg/mg creatinine)	137	151	94.8	432	763	531	260	***	n.s.	n.s.	n.s.

Abbreviations are: CON = control diet ; CE = thymol on celite 545 coarse as carrier; AL = thymol on alphacel non-nutritive bulk as carrier; ME = microencapsulated thymol and SE = standard error.

(1) thymol was not detected in the control treatment; determined concentration in plasma and urine includes both the free and conjugated parent compound; (2) n.s. = non-significant ($P>0.1$); T = trend ($P<0.1$); * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; (3) I = orthogonal contrast 500 mg/kg vs. 2000 mg/kg; II = orthogonal contrast CE vs. AL; III = orthogonal contrast CE vs. ME and IV = orthogonal contrast AL vs. ME.

DISCUSSION

EO concentration in digesta and antibacterial effects

The gastric EO concentration (Table 4 and 6) was higher than the critical concentration indicated by Michiels *et al.* (in press) (Chapter 2) or antimicrobial doses used by Si *et al.* (2006) and therefore antimicrobial effects were anticipated. However, the 2000 mg/kg diets did not reduce the number of any bacterial group compared to CON in this segment (Table 2 and 4). Further analysis of the data showed that there were large differences between animals, with a 1.5 and more than 2-fold range in the concentrations in gastric digesta for experiment I and II, respectively. Pearson correlation coefficients between concentration and number of bacteria in gastric digesta were calculated for each experiment. No significant (negative) correlations were found, so there are no indications for any antimicrobial activity in the stomach. Probably, the higher dry matter content of the gastric digesta as compared to the *in vitro* medium used in the study of Michiels *et al.* (in press) (Chapter 2) (app. 300 *vs*. 100 g/kg), can explain this lack of effect. The detrimental effect of dry matter on the antibacterial activity of EO was evidenced in food matrices (Smith-Palmer *et al.*, 2001). The mean EO concentration in S1 and S2 for the 2000 mg/kg treatments did not exceed 24 mg/kg, which is considered far too low to provoke a direct antimicrobial effect. Nevertheless, in the SI of animals fed CE2000 the bacterial load was reduced. These animals showed a lower feed intake (pen level) and smaller growth rates ($P<0.1$), which is obviously in contradiction to the effects usually observed in animals fed antimicrobial growth-promoters. A reduction in the number of bacteria in the other 2000 mg/kg and 500 mg/kg treatments has not been observed. Additionally, adaptation of bacteria could also have contributed to the lack of effect. Bacteria became tolerant to these EO when grown at sub-lethal concentrations (Di Pasqua *et al.*, 2006; Ultee *et al.*, 2000) and perhaps this might have been affecting the microbial ecology in our experiments. To conclude, based on our findings there is no conclusive evidence that these feed inclusion rates of carvacrol and thymol can reduce the bacterial

load (main bacterial groups quantified by plating onto selective media) in the foregut of piglets. Few data are available in literature; trials have been performed with pure compounds, mixtures and herbal extracts with lower and higher doses than in our trials. In none of these experiments a significant reduction of the foregut bacteria was observed (herbal extracts, 7500 mg/kg, Namkung *et al.*, 2004; Xtract, 150-300 mg/kg, Manzanilla *et al.*, 2004; herbal extract mixture, 700-1400-2100 mg/kg, Oetting *et al.*, 2006). Very recently Janczyk *et al.* (2008) found that supplementation of pig diet with thymol (10,000 mg/kg) caused clear changes in the small intestinal microbial community, as assessed by PCR.

Obviously, the low concentrations of the EO in the gut digesta found in our trials, were due to fast absorption in the proximal GIT, as can been seen in Fig. 1. However it has to be said, that there were large differences between animals. Absorption in the stomach was influenced by the dry matter content of the gastric digesta, since we found for both experiments a negative correlation between these two variables (exp. 1; r=-0.62, $P<0.01$ and exp. 2; r=-0.47, $P<0.01$). More fluid gastric contents can enhance the solubilization and emulsification of the EO and consequently absorption and/or gastric emptying with the liquid phase. It was found that formulation type (experiment II) did not affect the kinetics of the EO in the GIT. In the study of Piva *et al.* (2007a) animals were fed either an unprotected or a microencapsulated mixture of sorbic acid and vanillin. They reported the ratio concentration in stomach (on DM basis) to concentration in proximal and distal SI. For animals fed the microencapsulated ingredients, these ratios were 0.44-0.48 and 0.35-0.55 for proximal and distal SI respectively. In animals fed the unprotected ingredients, these fractions were below 0.02. In our study, these ratios were similar for all treatments and below 0.1. There is no explanation why the microencapsulation did not retard the release (and absorption) of thymol in experiment II. A single batch of microencapsulated thymol was made for this trial and likely the technical preparation was not fully successful. The application of appropriate *in vitro* techniques to test the release properties of formulations in advance to *in vivo* research is prompted.

Improving gut health

In experiment II, thymol fed animals showed lower number of IEL in the small intestinal mucosa (for some treatments with significance, $P<0.05$; see also orthogonal contrast I in Table 5). IEL are T-lymphocytes, with the majority of the cytotoxic CD8+ phenotype. Thymol has shown to reduce the biosynthesis of prostaglandins through its cyclooxygenase inhibitory activity. The IC50 value against cyclooxygenase-1 was 0.03 mg/L and was comparable to that of indomethacin (Marsik *et al.,* 2005). Prostaglandins are mediators of inflammatory responses, hence thymol might have downregulated mucosal inflammatory responses and concomittantly the recruitment and migration of T-lymphocytes to the villus epithelium. The reduction in number of IEL did not depend on formulation type and dose. Presumably, the thymol small intestinal concentrations as observed for all treatments (Table 6) were high enough to provoke this effect. A reduced number of IEL can be assumed to be a beneficial effect as a positive relationship between the number of IEL in the small intestinal mucosa and its turnover rate has been reported (Guy-Grand *et al.*, 1998). Concurrently, the V/C ratio at S2 was higher in carvacrol and thymol fed animals (exp I, $P<0.05$; exp II, $P<0.1$). A higher V/C ratio is associated with a slower enterocyte migration rate and concomitant turnover rate of the epithelia. This situation can result in improved digestive and absorptive capacities of the SI as fully mature enterocytes have a higher activity of brush border enzymes. This change in V/C ratio was accompanied with enlarged villi or reduced crypts. Especially, for the 500 mg/kg treatments it was seen that villi at J2 were longer, although not significant. Obviously, there is a clear relationship between the 12 days post-weaning feed intake - all but one (ME500) of the 500 mg/kg treatments gave a higher feed intake compared to CON (from +12.4 to +44.7% on pen level) - and the length of villi at day of slaughter. It is well established that immediate post-weaning feed intake and the presence of digesta in the gut *per se* stimulate the development/adaptation of gut histo-morphology and consequently leads to a less severe villi atrophy or better recovery of villi structure (Pluske *et al.*, 1997). Further, thymol supplementation notably resulted in more appetite compared to carvacrol as is

evidenced in experiment I. This difference was also observed in a previous unpublished latin-square experiment wherein the 6 h feed intake after supplying an experimental feed (2000 mg/kg carvacrol, thymol or control feed), preceded by giving control feed and temporarily fasting of the animals, was followed up. Adverse effects of 2000 mg/kg EO on appetite were greater for carvacrol than for thymol.

In addition, different results indicate that gut health was improved in treatments THY2000 and ME2000. Higher numbers of both streptococci and lactobacilli were found in S1 and S2 for THY2000. ME2000 had lower numbers of coliforms in all three sampling sites and higher numbers of streptococci in all three sampling sites and lactobacilli in S2. Hence, it is fair to postulate that these treatments resulted in a higher ratio of lactic acid bacteria/coliforms in the SI. This ratio is considered to be an indicator of a healthy gut and pathogen resistance (Reid and Hillman, 1999). Lactic acid bacteria produce lactic acid which results in lower pH values (Jensen and Mikkelsen, 1998), possibly suppressing the proliferation of potentially pathogenic G- bacteria. In case the number of total anaerobic bacteria are unaltered (as observed for most treatments here) or reduced, a shift in the ratio lactic acid bacteria to coliforms in favour of the former group can be considered as a positive effect. It was seen that the gastric pH for the treatments THY2000 and ME2000 was substantially lower than the respective CON diet (Table 2 and 4). A lower gastric pH would increase the barrier function of the stomach towards acid susceptible bacteria like coliforms and favour the growth of acid tolerant bacteria like lactic acid bacteria, a phenomenon that can be observed even more caudally in the SI (Canibe and Jensen, 2003). So, the higher ratio lactic acid bacteria/coliform found in S2 for both diets can be due to the lower pH in the stomach rather than a direct effect of thymol on bacteria in S2. An increased retention time of gastric digesta can eventually result in a lowered pH. This was hypothesised in the trial of Manzanilla *et al.* (2004). The authors argued that capsaicin was responsible for the slower gastric emptying, and this resulted finally in a higher ratio lactobacilli/coliforms in the distal SI. Relaxant effects on smooth and skeletal muscle have been

found for carvacrol (Aydin *et al.*, 2007; Boskabady and Jandagh, 2003) and thymol (Beer *et al.*, 2007; Szentandrassy *et al.*, 2003) and this could suggest a role in gastric motility. However, in our trial the gastric dry matter weight of all treated animals was comparable (or lower) compared to control animals, although having similar BW. This would indicate that gastric emptying was not affected (or enhanced) in the treated animals. Likewise, an effect on gastric motility cannot be confirmed here. In both experiments, a significant ($P<0.01$) positive correlation between gastric fresh weight content and pH (exp. 1; r=0.57 and exp. 2; r=0.73) has been found. It indicates that smaller amounts in stomach became more acidified. Probably, here gastric pH was more related to gastric fill than to other factors, like retention time and feed buffering capacity. Why this was only observed for THY2000 and ME2000 is not clear.

CONCLUSION

Supplementing feeds for newly weaned piglets with 500 mg/kg carvacrol and thymol may enhance post-weaning feed intake and result in longer villi (non-significant). These two EO at both 500 and 2000 g/kg feed did not reduce the number of bacteria or their metabolites in the foregut, probably as a result of the high dry matter content of gastric digesta and the fast absorption in the proximal SI. Formulation type (soybean oil, inert carriers and microencapsulation) did not alter the release and absorption of the EO in the GIT. Carvacrol and thymol diets reduced to a variable extent the number of IEL and concurrently the V/C ratio at the distal SI was higher. Both effects indicate that gut health was improved. In view of the limited number of animals in these trials, confirmation of these findings is warranted.

ACKNOWLEDGEMENTS

The authors wish to thank SODA Feed Ingredients (Monaco) for kindly providing the microencapsulated thymol. Part of this work was financially supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT), Brussels, Belgium. The authors are also grateful to C. Vermander, A. Maes, E. Turtelboom, S. Coolsaet, S. Lescouhier and Y. De Bock for skilful technical support.

CHAPTER 5

EFFECT OF THE ESSENTIAL OILS THYMOL, *E*-ANETHOLE AND *E*-CINNAMALDEHYDE ON ACTIVE NUTRIENT ABSORPTION AND STIMULATED CHLORIDE SECRETION IN THE ISOLATED PIG GUT

Part of this Chapter is presented in

Michiels, J., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (submitted). Thymol and *trans*-cinnamaldehyde reduce active nutrient absorption and stimulated chloride secretion in the pig jejunal Ussing chamber model. Livestock Science.

CHAPTER 5

EFFECT OF THE ESSENTIAL OILS THYMOL, *E*-ANETHOLE AND *E*-CINNAMALDEHYDE ON ACTIVE NUTRIENT ABSORPTION AND STIMULATED CHLORIDE SECRETION IN THE ISOLATED PIG GUT

ABSTRACT

The essential oils thymol, *E*-anethole and *E*-cinnamaldehyde have been shown to exert antimicrobial properties against commensal and pathogenic bacteria of the porcine gut. Therefore, they have gained interest for use in animal feeds. However, distinct biological activities are reported which can interfere with the absorptive and secretory functions of the gut epithelia. Hence, we studied the effect of these compounds on sodium-dependent D-glucose and L-alanine absorption and stimulated chloride secretion. Stripped mid-jejunal tissue of fully weaned piglets was mounted in modified Ussing chambers. The increase in short-circuit current (ΔIsc) to mucosal addition of 16 mmol/L D-glucose and 16 mmol/L L-alanine, with or without pre-incubation with 0.2 and 1.0 mmol/L thymol or *E*-cinnamaldehyde and 0.2 and 0.5 mmol/L *E*-anethole was determined. Accordingly, the effect on stimulated chloride secretion by secretagogues was measured. Thymol and *E*-cinnamaldehyde reduced the Isc response to nutrients and secretagogues in a dose-dependent manner. ΔIsc to D-glucose for thymol at 0.2 and 1.0 mmol/L was respectively 73.7 ± 7.9 (P>0.05) and 23.8 ± 7.4 (P<0.001) compared to 80.1 ± 7.4 μA/cm^2 for control and to L-alanine respectively 20.6 ± 2.3 (P=0.01) and 5.9 ± 2.4 (P<0.001) compared to 33.7 ± 2.6 μA/cm^2 for control. *E*-cinnamaldehyde showed only significant effects at 1 mmol/L. Pre-incubation with 0.5 mmol/L *E*-anethole did not alter

the response to mucosal D-glucose. The inhibitory activity of thymol and *E*-cinnamaldehyde on active nutrient absorption could compromise intestinal digestive functions. The reduction in secretory response to cAMP/cGMP mediated VIP and theophylline was greater than for Ca^{2+} mediated 5-HT and carbachol. Chloride secretion induced by 5-HT and theophylline was also affected by 0.5 mmol/L *E*-anethole. A reduction of induced secretion might be beneficial in cases of excessive chloride secretion. Additional investigations are urged to confirm these findings and eventually elucidate the mechanisms behind.

KEYWORDS: Essential oils, Ussing chamber, sodium-dependent nutrient absorption, chloride secretion, diarrhoea, pig

INTRODUCTION

Essential oils (EO) with well documented antimicrobial properties like thymol, *E*-cinnamaldehyde and to a lesser extent *E*-anethole (*e.g*. Michiels *et al.*, 2009 – Chapter 2) have gained renewed interest for application in animal feed, mainly due to constraints on the use of antibiotics and metals as AMGP. The mode of action of the AMGP is not fully understood but there is evidence that they work in part by decreasing the overall numbers and/or specific bacterial species or their metabolic activities in the GIT (Anderson *et al.*, 1999; Corpet, 2000; Decuypere *et al.,* 1991; Gaskins *et al.*, 2002; Visek, 1978).

In particular, Dierick *et al.* (1981) showed in an isolated loop pig model that Virginiamycin (50 mg/L in solution) improved the absorption of free amino acids. This feature of Virginiamycin may partly explain the observed higher ileal nutrient digestibility and the improved feed conversion in pigs (Decuypere et al., 1991). Little is known about the effects of EO on nutrient absorption and secretion. Kreydiyyeh *et al.* (2003) demonstrated in a rat perfusion model that 0.05% aniseed EO (76.7% *E*-anethole) significantly increased jejunal glucose absorption and this coincided with the stimulation of the Na^+-K^+-ATPase activity, the driving force for secondary active transport, in jejunal homogenates. On the contrary, eugenol (app. 5.2 mmol/L) and cinnamaldehyde (app. 7.5 mmol/L) reduced significantly the L-alanine absorption, which was attributed to the inhibition of basolateral Na^+-K^+-ATPase activity (Kreydiyyeh *et al.*, 2000). In the Ussing chamber model, Beesley *et al.* (1996) showed that mucosal peppermint oil (1 and 5 mg/mL) inhibited sodium-dependent glucose absorption, but not acetylcholine stimulated chloride secretion. In contrast, serosal peppermint oil at 1 mg/mL reduced chloride secretion induced by acetylcholine without affecting the effect of glucose.

Taking together, these results for EO are quite conflicting and were in fact solely obtained in rat models. Any effect on active nutrient absorption might interfere with feed digestibility; obviously an improved capacity for nutrient absorption can be considered beneficial. The preventive effect of zinc

on diarrhoea in newly weaned piglets is partially due to the attenuation of chloride secretion caused by bacterial toxins (Carlson *et al.*, 2004 and Carlson *et al.*, 2006). So, a reduction of chloride secretion stimulated by secretagogues, as examplified by Beesley *et al.* (1996) with peppermint oil, could be indicative for a similar mode of action. This led us to conclude that it is of high interest to study the effect of thymol, *E*-anethole and *E*-cinnamaldehyde on active nutrient absorption and induced chloride secretion in the pig intestine, as these compounds may come into contact with the intestinal mucosa after oral application. Moreover, cytotoxic and apoptotic effects by EO against intestinal Caco-2 and IPEC-1 cell cultures were found by respectively Dusan *et al.* (2006) and Bimczok *et al.* (2008), which could compromise the epithelial absorptive and secretory function. In the research presented here, the Ussing chamber technique was used.

MATERIALS AND METHODS

Animals and tissue

Each of the series of experiments included 5 to 6 fully weaned piglets with an average live weight ± standard deviation of 16.1 ± 2.5; 15.9 ± 1.7; 18.1 ± 0.1; 15.6 ± 0.7 and 15.6 ± 1.1 kg for series 1, 2, 3, 4 and 5 respectively. Piglets ((Landrace x Large White x Seghers Hybrid synthetic line) x Piétrain) were weaned at 25 days of age. After weaning piglets received a weaner (2 weeks) and a starter diet *ab libitum* until day of euthanisation. Both diets were without AMGP. The composition of the starter diet was (in g/kg): 667 maize, 283 soybean meal, 10.3 soybean oil, 3.6 HCl-lysine, 0.8 DL-threonine, 0.4 L-tryptophane, 6.5 sodium chloride, 12.5 monocalcium phosphate, 14.3 calciumcarbonate and 2.5 vitamin and trace element mixture. Water was available *ad libitum*. The euthanasia procedure included electric stunning followed by exsanguination. Immediately thereafter, a midline abdominal incision was made and the complete GIT was removed. A segment of 20 cm, distal from mid-small intestine was taken, rinsed with a Ringer's buffer solution and then placed in an oxygenated Ringer's

buffer solution at 38°C. The Ringer's solution (pH 7.4) contained (in mmol/L): 115 NaCl, 25 $NaHCO_3$, 0.4 $NaH_2PO_4.H_2O$, 2.4 $Na_2HPO_4.2H_2O$, 5 KCl, 1.2 $CaCl_2.2H_2O$, $MgCl_2.6H_2O$ and 6 D-glucose. The segment was opened longitudinally along the mesenteric border. The epithelium was stripped of its serosal and muscle layers and then mounted in 6 modified Ussing chambers (acrylate polymer) (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany). To confirm correct tissue preparations, histological assessments were performed according to Van Nevel *et al.* (2003). Edge damage was minimised by placing silicon sheets on both sides of the tissue. The Ussing chambers had an opening area of 1.07 cm^2. All tissues were mounted within 15 minutes following euthanisation. A volume of 6.5 mL of the following buffer solution (pH 7.4) was added at the serosal side of the epithelium (in mmol/L): 115 NaCl, 25 $NaHCO_3$, 0.4 $NaH_2PO_4.H_2O$, 2.4 $Na_2HPO_4.2H_2O$, 5 KCl, 1.2 $CaCl_2.2H_2O$, $MgCl_2.6H_2O$ and 12 D-glucose. On the mucosal side, 12 mmol/L D-glucose was replaced by 12 mmol/L mannitol. The temperature of buffer solutions was held constant at 38°C and mixed and gassed with a 95% O_2 and 5% CO_2 mixture by a gas lift system.

Measurement of electrophysiological parameters

Electrical measurements were obtained by a six-channel microcomputer-controlled voltage/current clamp (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany). Two 3 mmol/L KCL agar bridges were positioned near each surface of the tissue (< 3mm) and connected to Ag/AgCl electrodes by 3 mmol/L KCl solution for the measurement of the transepithelial potential difference (PD). Two distant 3 mmol/L KCL agar bridges were connected with Ag/AgCl electrodes by 3 mmol/L KCl solution for passing current through the tissue. Before mounting the epithelial tissue in Ussing chambers, the voltage/current clamp was corrected for fluid resistance. The electrophysiological measurements were started under open circuit conditions. The transepithelial resistance (Rt; $\Omega.cm^2$) was determined from voltage deflections (PD; mV) in response to bipolar 50 μA current pulses generated for 200 ms. The measured PD and corresponding Rt, calculated by the

Ohm's law were given every 6 s. After an equilibration period of 15 minutes, tissues were short-circuited. The short-circuit current (Isc; $\mu A/cm^2$) was assumed to be an indirect measure of net transcellular electrolyte movement. The Isc was recorded continuously and data were transferred to a computer with the software package Clamp version 2.14 (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany).

Experimental design

Thirty-five minutes after mounting the tissues, experimental protocols were started. A stock solution of the respective EO was added to the mucosal side with increasing final concentrations in the bathing media; 0, 0.2 and 0.5 (only *E*-anethole; 117870, Sigma-Aldrich nv/sa, Bornem, Belgium) or 1.0 mmol/L (thymol; T0501, Sigma-Aldrich nv/sa and *E*-cinnamaldehyde; 96320, Fluka, Bornem, Belgium). Stock solutions (5 μL) were prepared in absolute ethanol and added to each chamber. Preliminary experiments showed that the addition of 5 μL absolute ethanol had no effect on basal values. The highest concentration tested for *E*-anethole was 0.5 mmol/L, since this corresponds approximately to its maximum water solubility (Terpene Consortium, 2002). Each concentration was tested in two chambers. Five minutes after EO addition the change in basal Rt (ΔRt_{BASAL}; $\Omega.cm^2$) and basal Isc (ΔIsc_{BASAL}, $\mu A/cm^2$) were recorded. In series 1, 2 and 3, respectively thymol, *E*-anethole and *E*-cinnamaldehyde were tested for their effect on D-glucose absorption and stimulated chloride secretion by 5-HT (5-hydroxytryptamine or serotonine; H9523, Sigma-Aldrich nv/sa) and theophylline (T1633, Sigma-Aldrich nv/sa). Therefore, at 55 min, 16 mmol/L D-glucose was added to the mucosal side and simultaneously 16 mmol/L mannitol was added to the serosal side in all chambers. Twenty minutes later, 5-HT (0.1 mmol/L) was added to the serosal side. Finally, 20 min after adding 5-HT, 5 mmol/L theophylline was added bilaterally. In series 4 and 5, thymol and *E*-cinnamaldehyde respectively were tested for their effect on L-alanine absorption and stimulated chloride secretion by VIP (vasoactive intestinal peptide; V3628, Sigma-Aldrich nv/sa), carbachol

(LANSL06674.06; VWR, Leuven, Belgium) and theophylline. At 55 min, 16 mmol/L L-alanine was added to the mucosal side and simultaneously 16 mmol/L mannitol was added to the serosal side in all chambers. Twenty minutes later, VIP (0.1 µmol/L) was added to the serosal side, followed at an equal time interval before serosal addition of carbachol (1 mmol/L). Finally, 20 min after adding carbachol, 5 mmol/L theophylline was added bilaterally. The change in Isc (ΔIsc; µA/cm^2) due to addition of nutrient and secregatogues was calculated by subtracting the Isc before stimulation from the peak response after stimulation.

In two experiments (one with thymol and one with *E*-cinnamaldehyde); after termination of the experiment, tissues were pinned on cork, transferred to buffered formalin and processed for histological survey (Van Nevel *et al.,* 2003). In three experiments with thymol at 1.0 mmol/L from series 1, samples from the mucosal and serosal compartment (6 chambers) were taken at the end of the experiment (sampling 80 min after thymol addition) and the thymol concentration herein was determined (Michiels *et al.,* 2008 – Chapter 3).

Statistical analysis

Statistical analysis was carried out by the General Linear Model in SPSS (SPSS 15.0 program software; SPSS Inc., Chicago IL, USA) whereby EO concentration (α_i; i=0, 0.2 and 0.5 or 1.0 mmol/L) was included as fixed factor and animal (β_j; 5 to 6 piglets per series) as random factor. The model represents:

$$y = \mu + \alpha_i + \beta_j + \alpha_i \,.\, \beta_j + \varepsilon_{ij}$$

where y is the dependent variable, µ is the overall mean and ε_{ij} the residual error. If the interaction term $\alpha_i \,.\, \beta_j$ was not significant (P>0.05) it was excluded from the model. The type of sum of squares in the procedure was chosen to correct for unbalanced data. Treatment means (concentration) were compared with the LSD test. Results are presented as adjusted means ± standard error.

RESULTS

Histological assessments and thymol concentration

Histological investigations of the mounted jejunal tissue showed an appropriate stripping of the outer muscle layers and preservation of submucosa (data not shown). Post-experimental examination of tissues (two experiments), showed a similar appearance with maintenance of epithelial layer integrity in all treatments. Minimal sloughing of villus cells and oedema in the lamina propria could be observed in most tissues. In Fig. 1 a control tissue and a tissue bathed in 1 mmol/L *E*-cinnamaldehyde is shown. The mean mucosal thymol concentration, 80 minutes after thymol application (1.0 mmol/L; mucosal side) was 0.46 ± 0.03 mmol/L (n=6 chambers), which corresponds to approximately half of the initial concentration. The concentration in the serosal buffer was lower than 1.5 µmol/L.

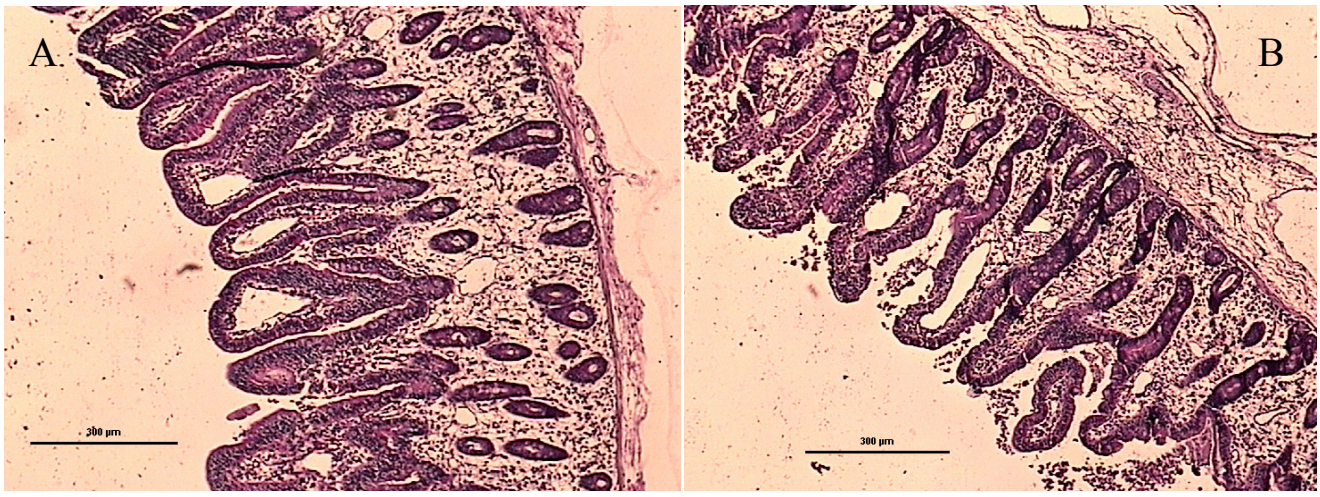


Fig. 1. Examples of histological sections of jejunal tissue after an experiment in the Ussing chambers (115 min) and stained with haematoxylin-eosin; A: control treatment, B: *E*-cinnamaldehyde, 1 mmol/L.

Effect on basal short-circuit current and tissue resistance

Table 1 represents the results of the Ussing chamber study. An example of electrical recordings is given in Fig. 2. Thymol (series 1 and 4) and *E*-cinnamaldehyde (series 3 and 5) at 0.2 mmol/L showed a significantly higher ΔIsc_{BASAL} compared to the control ($P<0.05$). It means that the basal short-circuit current was affected after addition of the EO. The Isc increase at 1.0 mmol/L was not different from the control. The addition of *E*-cinnamaldehyde to the mucosal buffer provoked a slight increase in tissue resistance (Rt) at both doses compared to the control ($P<0.05$).

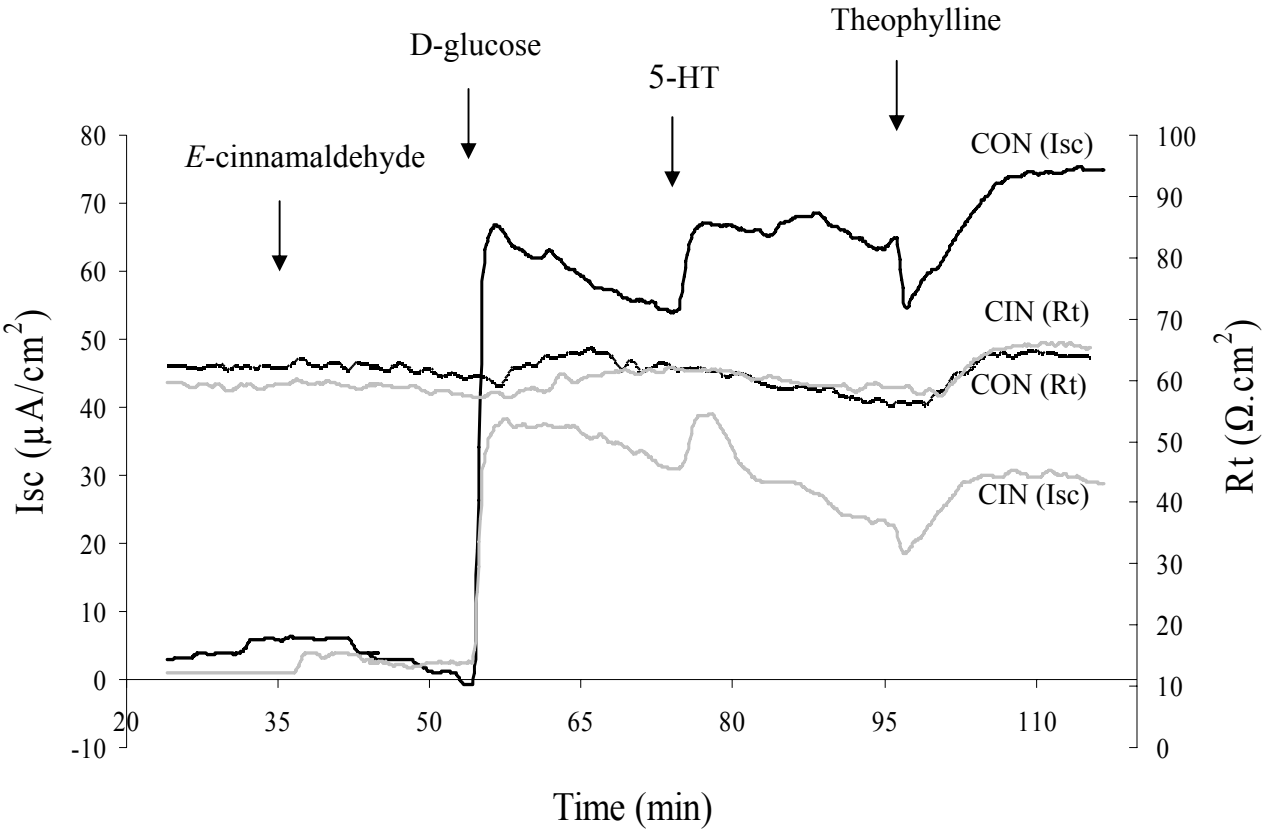


Fig. 2. Example of short-circuit current (Isc) and tissue resistance (Rt) recordings in response to mucosal addition of 16 mmol/L D-glucose at the mucosal side, 0.1 mmol/L 5-HT at the serosal side and 5 mmol/L theophylline bilaterally. The recordings of two Ussing chambers from Series 3 are shown: control treatment (CON, black lines) and pre-incubation with 1.0 mmol/L *E*-cinnamaldehyde added to the mucosal buffer (CIN, grey lines)

Effect on active nutrient absorption

Changes in short-circuit current after addition of D-glucose and L-alanine are a measure of sodium-coupled nutrient absorption. Neither, *E*-anethole at 0.2 and 0.5 mmol/L, nor thymol and *E*-cinnamaldehyde at 0.2 mmol/L affected sodium-dependent D-glucose absorption (Table 1). Thymol at 0.2 mmol/L reduced $\Delta Isc_{L\text{-}ALANINE}$ by almost 40% compared to the control ($P<0.05$). The highest concentration of thymol and *E*-cinnamaldehyde had clearly a great impact on active nutrient absorption ($P<0.05$). The Isc response to D-glucose for the thymol and *E*-cinnamaldehyde treatment was respectively 29.7 and 61.0% compared to that of the control, while the Isc response to L-alanine dropped further down to respectively 17.5 and 51.5%. The inhibitory effect of thymol exceeded by far the effect of *E*-cinnamaldehyde.

Effect on induced chloride secretion

All three EO reduced to a variable extent the Isc response to the secretagogues (Table 1). The chloride secretory response to neurotransmitters VIP and acetylcholine (and its mimetic carbachol) is mediated by elevated cAMP and intracellular Ca^{2+} levels respectively, which in turn activates chloride channels at the apical membrane resulting in chloride secretion (Barrett & Keely, 2000). Anion secretion induced by 5-HT is also via a calcium-dependent mechanism through binding to 5-HT_2 receptors located on enterocytes. However, *in vivo* this peptide also evokes the release of acetylcholine and non-adrenergic, non-cholinergic neutrotransmitters (most likely VIP) from enteric nerve endings. These effects may be quantitatively more important in the overall secretory response of 5-HT. Theophylline is a cAMP and cGMP phosphodiesterase inhibitor and will therefore inevitably result in an increase of intracellular levels of these second-messengers and subsequently chloride secretion. It is striking that for both thymol and *E*-cinnamaldehyde at 1.0 mmol/L the reduction in the Isc response as compared to the control was greater for the cAMP/cGMP mediated

secretagogues VIP (49.2 and 63.5% compared to control, respectively) and theophylline (39.2 and 65.0%; taken from series 1 and 3) than for the Ca^{2+} mediated secretagogues 5-HT (64.2 and 79.6%) and carbachol (64.3 and 91.8%). So, the inhibitory effect seems to depend on the intracellular signalling pathway. The lower response to theophylline found in series 4 and 5 as compared to series 1, 2 and 3 is most likely caused by the action of VIP, that already triggered cAMP in advance or due to secondarily down-regulation of chloride secretion by carbachol and is in accordance to Carlson *et al.* (2006).

Table 1. Response of tissue resistance (Rt, $\Omega.cm^2$) and short-circuit current (Isc, $\mu A/cm^2$) to increasing mucosal concentrations (mmol/L) of the EO thymol, *E*-anethole and *E*-cinnamaldehyde. Data are presented as adjusted means ± standard error [(1)]

	Thymol [(2)]			*E*-anethole			*E*-cinnamaldehyde		
	0	0.2	1.0	0	0.2	0.5	0	0.2	1.0
	Series 1			*Series 2*			*Series 3*		
Rt_{BASAL}	47.3 ± 3.1	48.6 ± 3.5	51.2 ± 3.3	57.9 ± 2.9	59.3 ± 3.1	60.5 ± 3.3	58.2 ± 3.4	54.0 ± 3.0	60.4 ± 3.1
ΔRt_{BASAL}	0.3 ± 0.5	0.2 ± 0.6	0.5 ± 0.5	0.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.4	-0.2 ± 0.5^{a}	2.2 ± 0.4^{b}	2.8 ± 0.4^{b}
Isc_{BASAL}	5.5 ± 3.3	13.0 ± 4.1	7.0 ± 3.3	-1.5 ± 2.2	-0.2 ± 2.5	-0.6 ± 2.6	12.4 ± 5.0	6.2 ± 4.8	12.5 ± 4.6
ΔIsc_{BASAL}	0.9 ± 1.2^{a}	5.9 ± 1.5^{b}	3.5 ± 1.2^{ab}	1.3 ± 0.5	1.1 ± 0.5	0.5 ± 0.5	0.8 ± 1.1^{A}	3.7 ± 1.1^{B}	2.9 ± 1.0^{AB}
$\Delta Isc_{GLUCOSE}$	80.1 ± 7.4^{a}	73.7 ± 7.9^{a}	23.8 ± 7.4^{b}	76.7 ± 6.7	72.8 ± 7.0	69.4 ± 7.8	69.0 ± 5.4^{a}	77.1 ± 4.9^{a}	42.1 ± 5.4^{b}
$\Delta Isc_{5\text{-}HT}$	12.0 ± 0.9^{a}	8.3 ± 1.1^{b}	7.7 ± 1.0^{b}	14.8 ± 0.7^{a}	13.0 ± 0.8^{ab}	11.9 ± 0.8^{b}	10.3 ± 1.1^{ab}	12.0 ± 1.0^{a}	8.2 ± 1.2^{b}
$\Delta Isc_{THEOPHYLLINE}$	25.0 ± 1.6^{a}	16.2 ± 1.8^{b}	9.8 ± 2.2^{c}	25.4 ± 1.2^{aA}	21.8 ± 1.3^{abB}	19.9 ± 1.4^{bB}	22.3 ± 1.5^{a}	20.9 ± 1.5^{a}	14.5 ± 1.5^{b}
	Series 4						*Series 5*		
Rt_{BASAL}	55.7 ± 2.7	56.3 ± 2.4	55.2 ± 3.2				51.4 ± 3.3	51.0 ± 2.9	49.9 ± 3.5
ΔRt_{BASAL}	-1.0 ± 0.5	-0.2 ± 0.5	-0.7 ± 0.5				-0.1 ± 0.4^{a}	1.7 ± 0.4^{b}	2.2 ± 0.4^{b}
Isc_{BASAL}	8.6 ± 3.0	7.2 ± 3.0	4.4 ± 3.0				1.1 ± 3.9	3.5 ± 3.7	8.1 ± 4.5
ΔIsc_{BASAL}	0.5 ± 0.4^{a}	3.3 ± 0.5^{b}	0.5 ± 0.5^{a}				0.4 ± 0.8^{a}	4.6 ± 0.8^{b}	1.8 ± 0.9^{a}
$\Delta Isc_{L\text{-}ALANINE}$	33.7 ± 2.6^{a}	20.6 ± 2.3^{b}	5.9 ± 2.4^{c}				41.0 ± 2.8^{a}	35.1 ± 2.6^{a}	21.1 ± 2.8^{b}
ΔIsc_{VIP}	6.5 ± 0.7^{a}	6.9 ± 0.7^{a}	3.2 ± 0.6^{b}				6.3 ± 0.7^{A}	5.5 ± 0.8^{AB}	4.0 ± 0.9^{B}
$\Delta Isc_{CARBACHOL}$	27.7 ± 1.9^{a}	23.6 ± 1.9^{a}	17.8 ± 1.8^{b}				41.9 ± 2.9^{A}	33.7 ± 2.9^{B}	38.5 ± 2.9^{AB}
$\Delta Isc_{THEOPHYLLINE}$	12.7 ± 0.7^{a}	11.3 ± 0.8^{a}	7.2 ± 0.7^{b}				13.4 ± 0.9	11.4 ± 0.9	11.3 ± 0.9

[(1)] The number of animals used per series was 5 to 6 with 2 tissues per animal and concentration; [(2)] Values per EO within a row with different superscripts are significantly different at $P<0.1$ (A,B,C) or $P<0.05$ (a,b,c)

DISCUSSION

We investigated the effects of thymol, *E*-anethole and *E*-cinnamaldehyde on active nutrient absorption and induced chloride secretion in the pig Ussing chamber model. In the Ussing chamber technique, substances can be added to the buffers in order to assess the direct effect on the physiology of the mounted tissue; however limitations of the model have to be taken into account (Boudry, 2005) and viability of the tissue is limited (Söderholm *et al.,* 1998). Surprisingly, when the thymol concentration in both buffers at the end of the experiment was determined, no thymol was retrieved in the serosal buffer and approximately half of the initial concentration was found in the mucosal buffer. In an Ussing chamber study with chicken jejunal tissue, it was shown that with increasing mucosal addition of thymol (0.013, 0.067 and 0.133 mmol/L) the amount retrieved in the serosal buffer relative to the total amount retrieved declined (from 17 to 1%) (Haselmeyer, 2007). In our study, the applied doses were higher and the fact that thymol was not detected in the serosal compartement is then in line with the data of Haselmeyer (2007).

We demonstrated that thymol and *E*-cinnamaldehyde affected sodium-coupled D-glucose and L-alanine absorption in a dose-dependent manner. To a variable extent, chloride secretion induced by several secretagogues was reduced as well. Cloride is taken up into the cell across the basolateral membrane via the Na^+, K^+, $2Cl^-$-cotransporter, geared by the basolateral Na^+-K^+-ATPase that establishes an inwardly directed sodium concentration gradient. So far, we found that all Na^+-K^+-ATPase driven processes investigated here were significantly inhibited. Inhibition was noticed by pre-incubation with 1.0 mmol/L thymol and *E*-cinnamaldehyde and with 0.2 mmol/L thymol; only for L-alanine, 5-HT and theophylline. Presumably, the effects in our study can be ascribed to a direct inhibitory effect towards the activity of the basolateral Na^+-K^+-ATPase. It should be stressed that the EO are lipophilic and therefore can enter epithelial cells in a passive manner and therefore can reach the basolateral membrane. Consequently, all the cellular activities and processes that are driven by

the sodium gradient established by the Na^+-K^+-ATPase are expected to be impaired at the enterocyte level. This assumption is in agreement with the work of Kreydiyyeh *et al.* (2000), at least in the case of *E*-cinnamaldehyde. These authors showed that cinnamaldehyde inhibited the activity of rat intestinal Na^+-K^+-ATPase in a concentration-dependent manner (IC50; 1.1 mg/mg protein jejunal homogenate). Accordingly, they found that cinnamaldehyde (app. 7.5 mmol/L) reduced significantly L-alanine absorption in a rat jejunal perfusion experiment (app. 40% of control). Despite the fact that we used a different model, another species and lower concentrations, our results for *E*-cinnamaldehyde do well agree with those results. Nevertheless, other mechanisms could be involved as well. An alteration of enterocyte membrane fluidity could compromise the efficacy of membrane-embedded transport proteins. In particular, the phenolic thymol and its isomer carvacrol have been shown to accumulate in bacterial membranes and affect their fluidity and permeability (*e.g.* Ultee *et al.,* 1999) and this would be consistent with the more pronounced effect of thymol as compared to *E*-cinnamaldehyde. Another possibility can be a direct interaction of the compounds with the apical transporters: the co-transporter SGLT-1 (sodium-glucose linked co-transporter 1) for D-glucose, the respective neutral amino acid transporter for L-alanine and distinct chloride channels; the cAMP/cGMP-dependent CFTR (cystic fibrosis transmembrane conductance regulator) and calcium-dependent CaCC (calcium-activated chloride channel). A competitive type of inhibition of SGLT-1 by quercitin glucosides and green tea polyphenols has been suggested by Ader *et al.* (2001) and Kobayashi *et al.* (2000) respectively. Data for thymol or *E*-cinnamaldehyde are lacking. A direct interaction with these transport proteins is however unlikely, because the apical transport of D-glucose, L-alanine and chloride is mediated by several different proteins. *E*-anethole (0.2 and 0.5 mmol/L) did not alter the rate of D-glucose absorption. On the contrary, Kreydiyyeh *et al.* (2003) demonstrated in a rat perfusion model that aniseed EO significantly increased jejunal D-glucose absorption. These authors used aniseed EO, which contained 76.6% *E*-anethole, at a concentration of 0.05%. The concentration of *E*-anethole in the perfusate approximates then 2.58 mmol/L. Therefore, discrepancy between the study of Kreydiyyeh *et al.* (2003) and ours can be related to concentration,

the contribution of other components in the aniseed EO, the model applied or species difference. Finally, in our study only active nutrient absorption was measured, while in *in vivo* sodium-dependent and independent glucose absorption takes place. Shortly after a meal, brush border membrane GLUT2 (glucose transporter 2) levels are increased and serves as a means of sodium-independent glucose absorption to cope with high luminal glucose concentrations (Kellett & Helliwell, 2000). Kreydiyyeh *et al.* (2003) argued that their observation could be explained by the stimulation of the basolateral Na^+-K^+-ATPase, the transporter that drives sodium-dependent glucose absorption. Perhaps, an effect on sodium-independent glucose absorption was involved as well.

Boudry & Perrier (2008) found a dose-dependent (0-0.1 mmol/L) increase in Isc after addition of thymol and cinnamaldehyde. This rise in Isc immediately followed EO addition and returned rapidly to basal values; it means that these EO provoke a transient secretory response of electrolytes. They found that the ionic basis was different; thymol induced Cl^- and HCO_3^- secretion via a nervous pathway, likely by nicotinic receptor stimulation and cinnamaldehyde induced only HCO_3^- secretion, probably by direct activation of epithelial nicotinic receptors. In our study, we applied higher doses and the changes in Isc after addition of 0.2 mmol/L thymol and *E*-cinnamaldehyde were smaller and also much less then the effects of secretagogues. We determined the change in Isc five minutes after EO addition, because an immediate Isc increase as in the study of Boudry & Perrier (2008) was not observed here. The change in Isc for the higher concentration (1.0 mmol/L) was in none of the cases significantly different from control. We assume that at the higher concentration a stimulation of anion secretion is counteracted by the inhibitory effects as mentioned above. Interestingly, the addition of *E*-cinnamaldehyde to the mucosal buffer provoked a consistent slight increase in tissue resistance. Tissue resistance has been related to tight junction integrity and paracellular permeability (Lallès *et al.*, 2004). Therefore, an increased tissue resistance can enhance the epithelial barrier function towards entry of luminal antigens and invading bacteria. It was suggested that the lateral enterocyte

space may be the preferential site of entry for several pathogenic bacterial strains (*e.g.* Wells *et al.*, 1998). Although the effect of *E*-cinnamaldehyde was rather small, it deserves further attention.

Thymol and *E*-cinnamaldehyde inhibited the secretory response to all 4 secretagogues, while *E*-anethole at 0.5 mmol/L reduced the response to 5-HT and theophylline. This could imply that the EO would be beneficial in cases of excessive chloride secretion that results in the clinical picture of diarrhoea, *e.g.* in response to toxins such as the cholera toxin or ETEC enterotoxins whose mechanisms of action involve the neurocrine mediators (Barrett & Keely, 2000). This is of particular importance for piglets post-weaning as they show a higher sensitiveness to secretagogues (Montagne *et al.*, 2007). Hence, thymol and *E*-cinnamaldehyde show a more or less similar anti-secretory effect to zinc as was shown in the Ussing chamber model by Carlson *et al.* (2006). However, chloride and water secretion cannot solely be considered as a patho-physiological event. Limited secretion also has a physiological function as it comprises the more thorough mixing of the digesta with fluid and enzymes and guarantees a continuous rinsing of the intestinal surface counteracting the colonization of (pathogenic) bacteria (Lodemann *et al.*, 2006). Therefore, it can expected that these EO will interfere with this basal function of chloride secretion as well.

In addition, EO have been shown to affect intestinal cell viability and causing apoptosis and necrosis. However, the concentration range for these effects varies according the report. Bimczok *et al.* (2008) found a IC50 of carvacrol towards pig intestinal IPEC-1 cells of 0.53 mmol/L (MTT assay), while according to Roselli *et al.* (2007), the highest non-toxic concentration towards the same cell line was 5.00 mmol/L (assessed by cell permeability) for both carvacrol and cinnamaldehyde. Carvacrol, thymol and eugenol at 0.37, 0.17 and 0.52 mmol/L had no cytotoxic effects after 24 h against differentiated Caco-2 cells, while at the higher dose (1.83, 0.80 and 2.5 mmol/L, respectively) the EO, except for thymol, led to general damage of the cells (apoptosis and necrosis) and loss of monolayer adhesion (Dusan *et al.*, 2006). Apoptosis could be a result of the binding of EO to the cell

surface hydrophobically and thereby triggering an oxygen burst (Suzuki *et al.*, 1985) or by their effect on mitochondrial membranes resulting in an impairment of the electron chain reactions and a pro-oxidant activity; as proposed by Bakkali *et al.* (2008). From these literature data; 0.20 mmol/L should be non-cytotoxic. Regarding 1.0 mmol/L, it cannot be decisively concluded whether this dose in our Ussing chamber study could have induced loss of cell viability and consequently interfered with the epithelial absorptive and secretory capacity. In the Results section, it was shown that there was minimal sloughing of villus cells (also in control treatment). Probably, exfoliation of these cells was preceeded by deteoration of cell function and concomitant absorptive and secretory capacity and this process was aggrevated by EO addition. The fact that thymol was not retrieved in the serosal buffer also points at possible cytotoxic effects.

CONCLUSION

We demonstrated that thymol and *E*-cinnamaldehyde impaired sodium-dependent absorption of D-glucose and L-alanine *in vitro* in a dose-dependent manner. Pre-incubation with 0.5 mmol/L *E*-anethole did not alter response to mucosal D-glucose. The reduction by thymol and *E*-cinnamaldehyde in secretory response to cAMP/cGMP mediated secretagogues VIP and theophylline was greater than for the Ca^{2+} mediated secretagogues 5-HT and carbachol. *E*-anethole, at 0.5 mmol/L affected chloride secretion induced by 5-HT and theophylline. Inhibition of the basolateral sodium-potassium pump (*E*-cinnamaldehyde) and modification of membrane fluidity (thymol) might be involved but additional investigations are necessary to confirm these findings and eventually elucidate the exact mode of action.

ACKNOWLEDGEMENTS

The authors are grateful to P. Vandecasteele, A. Ovyn, A. Maes, E. Turtelboom and K. Taes for their skilful and dedicated technical support. Further, the introduction and assistance to the Ussing chamber technique by Dr. Gerhard Schultheiß (Institut für Veterinär-Physiologie, Justus-Liebig-Universität Gießen) and Dr. Ulrike Lodemann (Institut für Veterinär-Physiologie, Freie Universität Berlin) is highly appreciated.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PROSPECTS

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PROSPECTS

EO for use in pig nutrition has deserved a lot of attention during the last decade, with special emphasis to their antimicrobial properties. Despite an overwhelming amount of *in vitro* data concerning the latter, few *in vivo* trials with pure compounds have been published. Moreover, results varied according to experiment and a substantial reduction of the bacterial load in the foregut, like with other alternatives for AMGP (*e.g.* Dierick *et al.*, 2002b), has not been demonstrated yet. Therefore, it was of great interest to study the effect of these EO on gut bacteria and functionality in the pig and to understand possible pitfalls that arise when they are applied *in vivo*. In this thesis, 5 pure components were selected based on literature data and were investigated. It was preferred to work with pure compounds rather that plant EO, because: [1] biological activities observed can then inherently be related to the compound applied; [2] the composition of a plant EO can differ a lot according to factors specified in Chapter 1 and [3] requirements for authorisation (technical dossier to EFSA) are mainly addressed to single substances; registration of complex mixtures, in casu plant EO, can be problematic because of quality demands for composition whereby all active substances, impurities/contaminants and excipients should be quantitatively reported.

Antimicrobial activity and stability

The *in vitro* antimicrobial activity against the main components of the pig gut flora was established by means of simulations of the fermentation present in the different sections of the GIT (Chapter 2).

Samples from these experiments were taken to determine the stability/degradation in the respective section of the GIT (Chapter 3). Fig. 1 resumes these findings.

The isomers carvacrol and thymol showed similar antimicrobial activities and can be considered to be non-selective. In addition, they will not be degraded along the proximal GIT. From former research at our laboratory we concluded that a concentration that gives 1 $\log_{10}$ CFU/mL reduction in this type of *in vitro* batch incubations can result in appreciable reductions of the gut flora *in vivo* (Dierick *et al.,* 2002b). Considering the results for carvacrol and thymol, this would equal a dose of approximately 400 mg/L to reduce the coliforms in the SI and 400-550 mg/L to reduce the lactobacilli in the stomach and the SI. These concentrations are well below their water solubility and are somewhat in the same order of MIC values against various bacteria (Table 2, Chapter 1) and in line with the results of Si *et al.* (2006b). As both compounds were most effective against the dominant flora components like lactobacilli and streptococci in the proximal sections of the GIT and in an acidic environment, they must be well suited to reduce total bacterial load cranially and thus act as a growth-promoter. Nevertheless, the doses needed to obtain these effects are still rather high (> 400 mg/L). In two *in vivo* trials, we supplemented weaned piglets with 500 and 2000 mg/kg carvacrol or thymol (Chapter 4B). A higher inclusion level was not tested because of adverse effects on palatability (clearly illustrated in the choice-feeding study, Chapter 4A). With this dose, acute oral toxicity is not expected. If, for example piglets weigh 10 kg and consume 300 g feed a day, then an inclusion of 2000 mg/kg EO gives a daily EO intake of 60 mg/kg BW. This is well below the LD50 values for rodents (p58, Chapter 1). Theoretically, if the diluting effect of water, saliva and gut secretions is taking into account, 2000 mg/kg will result in gastric and small intestinal concentrations of approximately 600 and 200 mg/kg, respectively. This 600 mg/kg for stomach coincided more or less with what we found *in vivo*, but antibacterial effects were not present. This can be explained by the higher dry matter content of gastric digesta as compared to the *in vitro* medium used. Smith-Palmer *et al.* (2001) already demonstrated that a high dry matter content can abolish antibacterial

effects. The recovery in the SI was lower than theoretically deduced, due to absorption (see below). Probably, single phenolic EO - when administered in an economical and technical acceptable dose - will not be able to reduce the bacterial load *in vivo* and therefore act as a real growth-promoter. A realistic opportunity is to maximise synergism with organic acids or with antimicrobial peptides (endogenous or exogenous) (see also Chapter 1, 2.3). Both types of antimicrobial molecules stress/disrupt the integrity of the microbial membranes, as do phenolic EO. Therefore, their mode of action is not exclusive, rather they should work in conjunction as they have the same target site. More research should focus on this.

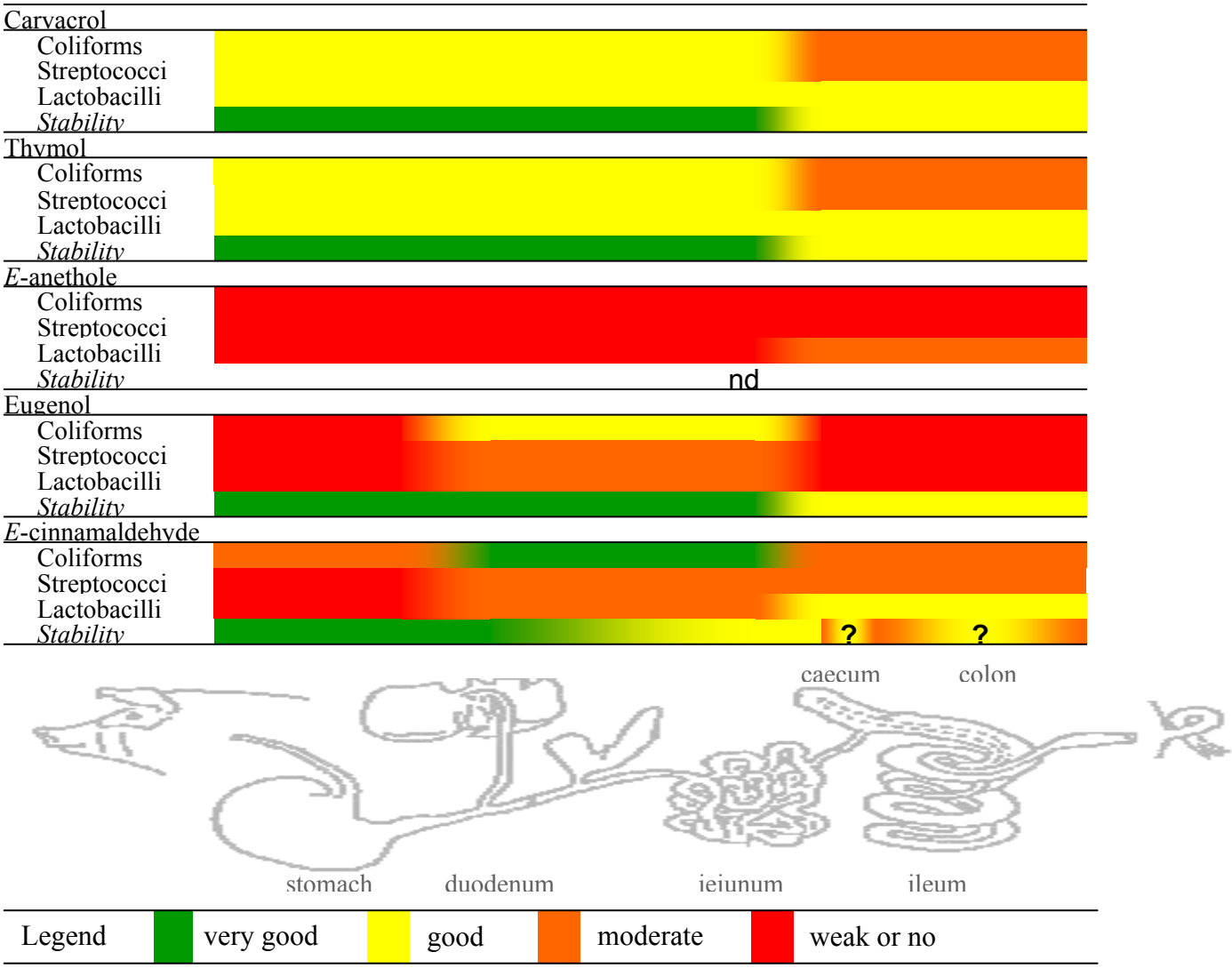


Fig. 1. Scheme representing the antimicrobial activity and stability of selected EO in *in vitro* simulations of the pig gut fermentation (for further details see Chapter 2 and 3).

Eugenol and especially *E*-cinnamaldehyde showed a selective antimicrobial spectrum and should induce a shift in the ratio lactobacilli/coliforms. The former was notably only active in jejunal simulations; and thus not in an acidic (stomach) and anaerobic (caecum) environment. No explanation can be given. *E*-cinnamaldehyde, at 100 mg/L, reduced substantially coliforms, which are mainly present in the distal SI. Coliforms are sometimes considered as indicator organisms for the presence of opportunistic pathogens. So, *E*-cinnamaldehyde holds promise to combat the pathogen load and secure intestinal health of pigs. We have not carried out *in vivo* trials with this compound, and only one preliminary report was found in literature (Andrés Elias *et al.,* 2007). Contrary to carvacrol and thymol, few studies have addressed the relationship between the biological activity and cinnamaldehyde structurally related compounds (Brackman *et al.,* 2008; Lee *et al.,* 2001; Lee *et al.*, 2007 and Lee *et al.,* 2008). Lee *et al.* (2001) found that some cinnamic acid derivates were equally effective as *E*-cinnamaldehyde against *E. coli*, but in contrast to the latter they did not affect the growth of *Bifodobacterium* spp. and *Lactobacillus* spp. In conclusion, these compounds were even more selective than *E*-cinnamaldehyde and could therefore balance the intestinal microbes further towards a healthy community (pathogen resistance, Reid & Hillman, 1999). More exploration of these findings is absolutely warranted. Interestingly, Niu *et al.* (2006) and Brackman *et al.* (2008) showed that *E*-cinnamaldehyde interfered with quorum sensing of bacteria. Evidence for quorum sensing mechanisms have been found for the rumen (Erickson *et al.,* 2002), but not for monogastrics. This needs further attention, and if so, again *E*-cinnamaldehyde is the molecule of choice.

However, as the compound owes its many biological functions to its reactive *α,β*-unsaturated aldehyde configuration (nucleophilic addition reactions), this provides the molecule also a low stability. Especially, thermal breakdown to benzaldehyde (Friedman *et al.,* 2000 and Gholivand & Ahmadi, 2008) should be of great concern for feed processing. In our research, it was partially degraded in *in vitro* small intestinal fermentations and its fate in caecal simulations could not be quantified due to analytical problems. It was clear that *E*-cinnamaldehyde showed weaker

antimicrobial activities in caecal simulations as could be predicted from its effect in jejunal simulations. Probably, it reacted here with matrix elements or was (bio)chemically transformed. We found cinnamic acid peaks on the chromatograms. However, detailed studies with more advanced analytical tools (for example, GC-MS) are necessary to understand its kinetics in the GIT. It is surprising, as *E*-cinnamaldehyde seems to be an interesting compound to modify ruminal fermentation patterns, that no data are available about its stability or appearance of metabolites in the rumen. This knowledge is needed to evaluate its efficacy and safety for the animal and its products. We found in the Ussing chamber model that *E*-cinnamaldehyde had a dose-dependent adverse effect on sodium-dependent nutrient absorption. However, these data can be questioned to some extent, since it is not known if this model is suited to test lipophilic compounds (although the concentration did not exceed its water-solubility). A study using a model more close to the *in vivo* situation (perfusion model) to confirm this finding is thus required. Nevertheless, Kreydiyyeh *et al.* (2000) already demonstrated that this compound was able to inhibit enterocyte Na^+-K^+-ATPase activity; the driving force for sodium-dependent nutrient absorption. Notably, many contemporary studies focus on elucidating the role of TRP channels in the animal's body and hence the full relevance of *E*-cinnamaldehyde as a potent agonist of TRPA1 is not understood. For example, Nozawa *et al.* (2009) showed that TRPA1 is highly expressed in enterochromaffin cells (EC) and that TRPA1 agonists activated EC function, such as the release of 5-HT. At least, this property of cinnamaldehyde (TRPA1 stimulation), explains partially its adverse effects on palatability in pigs fed high doses of this compound, which was clearly observed in preliminary trials at the laboratory.

There is a strong conviction that cinnamaldehyde-related compounds having the same (or even better) antimicrobial (and/or anti-quorum sensing) potential, without showing the disadvantages of the parent compound, are there to be found.

Fast absorption in the 'open system' GIT

An important observation in this thesis was that these EO were mainly and nearly completely absorbed in the stomach and the proximal SI when ingested (Fig. 1., Chapter 3 and Fig. 1., Chapter 4B). Fast absorption and rapid clearance of these compounds has also been reported in studies with humans and rodents. Hence, we found rather low luminal availabilities and lack of substantial antimicrobial activity in two *in vivo* trials with carvacrol and thymol (Chapter 4B). The highest concentration in the proximal and distal SI that we found in these trials was 40.8 mg/kg thymol (pig on diet AL2000) and 15.9 mg/kg thymol (pig on diet CE2000), respectively. Both pigs here were fed 2000 mg/kg thymol. It is surprising that many pig trials have been performed with particularly low inclusion rates of EO (see Table 5., Chapter 1); in only one study the dose was higher than in our research (Trevisi *et al.,* 2007). In none of these studies were the active ingredients dosed in the digesta. Nonetheless, the use of kinetic data of these agents is essential in order to predict effective feed inclusion rates and also to assess risks for tolerance/resistance development against these agents. Indeed various studies (Ultee *et al.,* 2000; Di Pasqua *et al.,* 2006) highlighted the potential for induced bacterial tolerance in the presence of sub-lethal doses of several EO. The bacteria lowered their membrane fluidity due to changes in the fatty acid and head-group composition of phospholipids. Adaptation of the gut microbiota was observed by Cardozo *et al.* (2004) and Report (2006). Many (soil)organisms can use these EO as carbon and energy source (Liotori, 1999), so they are biodegradable. It is not known whether and which gut bacteria can metabolise these EO. We found substantial degradation in caecal simulations, but did not research for underlying mechanisms. It might be possible that analogous mechanisms interfered with our observations made in the *in vivo* trials. The use of the EO *in vivo* should be well designed to avoid a continuous exposure of the gut flora to low levels of these compounds which will result eventually in microbial adaptation or important biodegradation and hence loss of efficacy or eventually favouring some microbes. Molecular techniques (*e.g.* PCR-DGGE) can be used to detect changes in the microbial population of

the gut. For example, in thymol fed animals (10000 mg/kg), the G- bacterium *Actinobacillus minor* was absent and in contrast, the G- enterobacterium *Citrobacter freundii* was present in most animals fed thymol (Janczyk *et al.,* 2008).

The notion that fast absorption can compromise an antibacterial effect gets particular relevance in cases where the bacterium is to be targeted in the ileum and caecum, *e.g.* the zoonotic pathogen *Salmonella enterica*. In the study of the kinetics of the EO in the GIT (Chapter 3), the estimated maximum concentration in the ileal digesta was 10, 4, 9 and 2 mg/kg for carvacrol, thymol, eugenol and *E*-cinnamaldehyde respectively (data not shown). The minimum reported MIC value against *Salmonella enterica* was 150, 57, 100 and 400 µg/mL for the same EO (Table 2., Chapter 1). It can be calculated that at least a 15, 14, 11 and 200-fold dosis in the feed than the one applied in the study might be necessary to achieve a concentration corresponding to the lowest MIC value. The feed inclusion level in the study for the EO was between 910 and 958 mg/kg. An alternative for higher feed inclusion levels is the application of appropriate release techniques. Active ingredients may be coated or microencapsulated (*e.g*. Meunier *et al.*, 2006 and Piva *et al.*, 2007) in order to delay the release of the active ingredients, but this makes it also more costly. Surprisingly, in our *in vivo* trial and for unknown reasons, micro-encapsulation of thymol did not give higher small intestinal concentrations compared to control. Another approach would be to covalently bind the compounds, for example as glycosides (which are actually present in plants in case of the phenolic compounds), but isolation or artificial synthesis is low-yielding and very costly. Additionaly, these technical formulations might provide more advantages: [1] protection from loss during feed processing and storage; [2] less distinct flavour and [3] less interaction with feed particles.

Effect on palatability and gut bacterial ecology and function

EO are frequently added to feed as flavours with the aim to affect the sensorial characteristics of the feed, mainly taste and smell. It means that they can affect palatability. We carried out a choice-feeding study to explore the effect of thymol and masking flavours on the animal's preference (Chapter 4A). 2000 mg/kg had a huge negative effect on appetite, but this was not so clear considering the ADFI in the two *in vivo* trials (Chapter 4B). A dose of 10000 mg/kg did reduce feed intake post-weaning (Trevisi *et al.,* 2007). There was an analogous difference with regard to 500 mg/kg thymol; no preference for this diet in the choice-feeding study but a higher ADFI in the *in vivo* trials was found. It seems that the results of the two studies do not exactly match. Interestingly, added flavours were able to mask partially the reduced preference for 2000 mg/kg in the choice-feeding study. So, if high inclusion rates are used, appropriate flavours can be helpful.

Effects on gut bacteria and function, as we found *in* and *ex vivo* are summarised in Fig. 2. Most AMGP target G+ bacteria (mainly lactic acid producing species) without affecting for example coliforms. A similar effect was shown for high doses of ZnO (Hojberg *et al.,* 2005). These additives have proven to be beneficial in terms of animal performance. From our observations - and in contrast to what we could expect based on the *in vitro* data – the EO carvacrol and thymol did not show a similar effect. Rather, they improved several indices that are related to a better gut health balance. Animals fed thymol showed lower numbers of IEL in the small intestinal mucosa, irrespective of formulation type or dose (for some treatments with significance, $P<0.05$; see also orthogonal contrast I in Table 5, Chapter 4B). It can be considered as a beneficial effect since a positive relationship between the number of IEL and mucosal turnover exists. Faster renewal of enterocytes lining the villi results in less mature enterocytes with less brush-border enzyme and absorptive activities and higher maintenance costs to restore epithelial integrity. A similar effect was demonstrated by Manzanilla *et al*. (2004), although they applied a low-dosed mixture containing carvacrol, cinnamaldehyde and

capsicum oleoresin. IEL are T-lymphocytes, with the majority of the cytotoxic $CD8^+$ phenotype. The number of IEL is increased by microbial colonisation (bacterial antigenic load) and to a lesser extent by dietary immunogenic factors. Research with germ-free animals showed underdeveloped intestinal lymphoid tissues, substantially decreased numbers of lymphocytes and low antibody concentrations; all which converted to the normal state when germ-free animals were associated with intestinal bacteria. Here, the same basal diet was used in all treatments, hence a dietary effect can be ruled out. Two hypotheses to explain the reduction in IEL by thymol are put forward:

[1] here, culturable bacterial groups were quantified on selective media and substantial changes in the number of gut bacteria were not detected. However, it is well established that with this approach the overwhelming diversity of the gut bacterial community cannot be fully characterised. Therefore, we speculate that it is not unlikely that thymol supplementation affected the growth of bacteria (*e.g.* Janczyk *et al.,* 2008) which was not detected here, but could possibly trigger the intestinal immune system differently. More investigations are needed to understand which immunogenic bacteria could have been affected by these EO, and

[2] carvacrol and thymol might have reduced the biosynthesis of prostaglandins through their COX inhibitory activity and hence downregulated (partially) inflammatory responses. Consequently, the (excessive) recruitment and migration of T-lymphocytes to the villus epithelium was restrained.

Overall, the fact that these EO can affect the immune response of the GIT warrants further attention.

Supplementation with carvacrol and thymol also resulted in a higher V/C ratio at the distal small intestine. A increased V/C ratio is associated with a slower enterocyte migration rate and concomitant turnover rate of the epithelium. For almost all 500 mg/kg diets a higher feed intake was found and this offers the opportunity to optimise the weaning transition period. The attenuating effect on stimulated (excessive) chloride secretion needs confirmation. Presumably, these EO are more useful

when animals are subject to gastro-intestinal disorders and/or unfavourable hygiene conditions (pathogen load).

These observations will contribute to our knowledge about the 'mode of action' of these EO, but many questions remain without answer. Based on the obtained data in this thesis and according to my opinion the following topics should be investigated with priority to better understand the interaction with gut bacteria and physiology:

[1] effects on gut microbiota (by plating and molecular techniques) and immune function (anti-inflammatory effects) by these EO and related compounds (especially cinnamaldehyde) in controlled-release formulations;

[2] effect on gut motility (see evidence for inhibitory effect on smooth muscle contractility);

[3] anti-oxidant effect (dose-response studies) towards feed components and gut tissue (phenolic compounds) and

[4] interaction with absorptive and secretory functions of the epithelium (confirmation of obtained results in perfusion model and eventually study of underlying mechanisms).

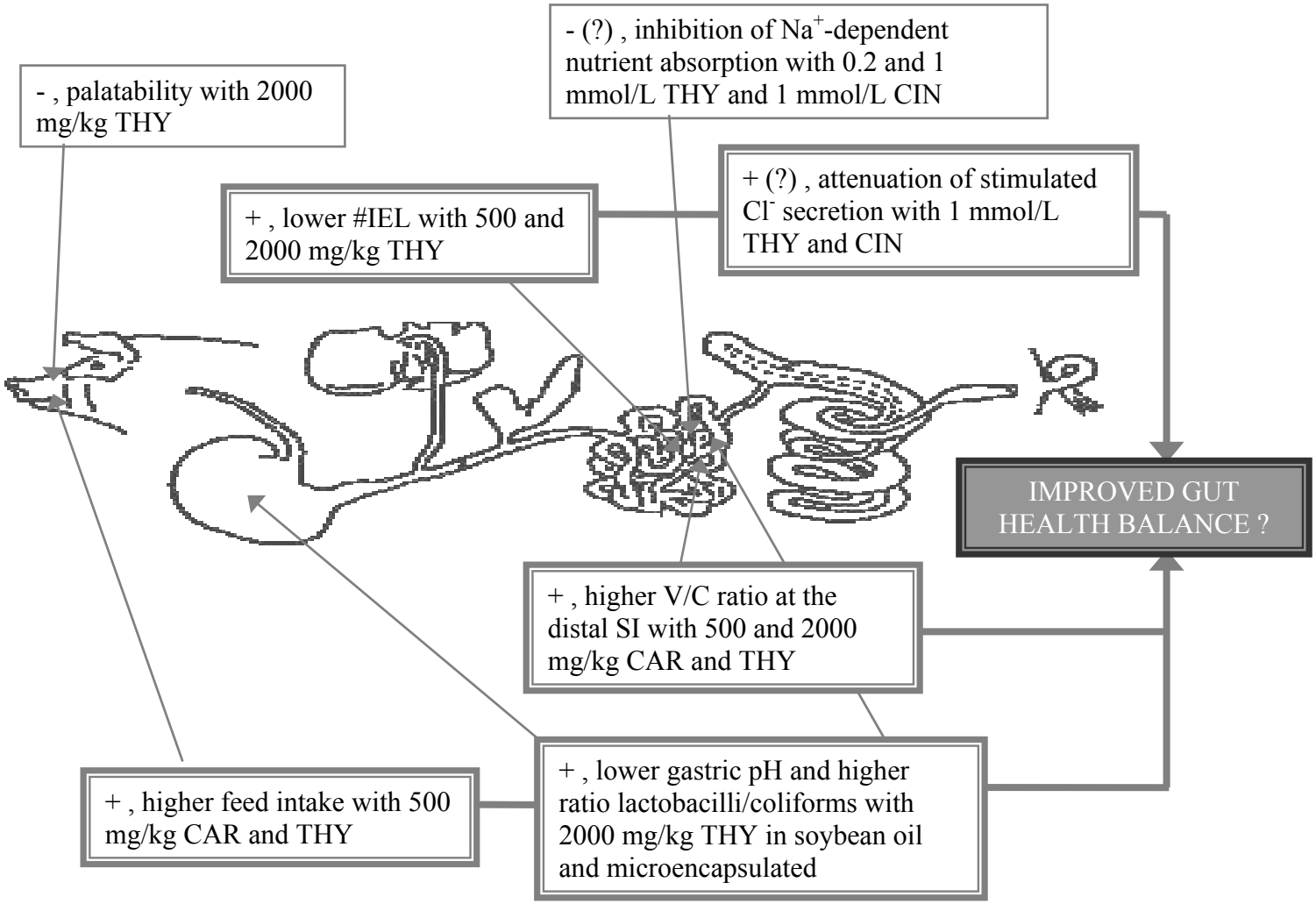


Fig. 2. Summary of *in* and *ex vivo* negative (-) and positive (+) effects of EO on palatability, feed intake and gut bacteria and function (for further details see Chapter 4A, 4B and 5). Some effects indicate that the gut health balance might be improved. Effects indicated with (?) need confirmation.

Potential risks and legal aspects

EO were studied here for their potential as alternative for the AMGP. The in-feed antibiotics were banned in the EU, for reasons of antimicrobial resistance, residue issues and negative consumer perception. Let's face these three topics for the EO; firstly antimicrobial resistance. There is evidence that bacteria can adapt to and degrade the EO (discussed above), but acquired genetic resistance has not been demonstrated. So far their efficacy is not ensured, but it is unclear whether this poses a risk for human health since their antimicrobial use for humans is limited. Intriguing is the work of Pumbwe *et al.* (2007). They demonstrated that sub-lethal cinnamaldehyde selected for multiple antibiotic resistance in *B. fragilis*. It means that there is a possibility that misuse of this EO can lead to bacterial resistance towards antibiotics. This has to be checked *in vivo*. With regard to the second point, residue issues are not anticipated (rapid metabolic conversion and clearance), but such research with high doses for pig has not been performed. In this respect, precaution is recommended. Thirdly, a major advantage of EO is that their consumer acceptance is high. People see this more as a 'natural remedie'. However, this is definitely not an alibi to declare that they are safe or not harmful.

Herbs and their extracts are increasingly being used (and informally marketed) not only as sensory additives, but also for other purposes, for example better growth or feed conversion, improved meat quality, and for prophylactic purposes. There are strong indications that such use will further increase in the future. As mentioned in Chapter 1, herbal extracts, flavours and EO now fall within the scope of EC Regulation 1831/2003. It means that for authorisation on the EU market, applications have to go through a set procedure. A key element hereby, is the evaluation of the technical dossier by the EFSA which should include proof of the claim and safety according to toxicological and tolerance tests. According to the claims made by the applicant and based on substantial proof of efficacy, herbal extracts will be classified either as technological (main action outside the animal) or as zootechnical (main action inside animal) feed additives. In both cases, for xenobiotic active

principles, a full toxicological data set will be required in order to guarantee safety for the animal, user, consumer and environment.

Essential oils???

To conclude, the *in vitro* studies showed the huge potential of carvacrol, thymol and *E*-cinnamaldehyde to modulate the composition of the gut bacteria of the pig. Contrary, in two trials with weaned piglets the supplementation with carvacrol and thymol at the doses applied did not reduce the bacterial load, most likely due to fast absorption of the active ingredient in the stomach and the proximal small intestine. Some effects indicate that the gut health balance might be improved, however the Ussing chamber study showed deleterious effects on active nutrient absorption. AMGP were an essential element of the enormous expansion and intensification of pig production systems during the last five decades. Essential oils might replace these AMGP partially, but in contrast to what their name suggests, they will not be essential. Besides, an essential oil is essential in the sense that it carries the distinctive scent, or essence, of the plant where it is derived from.

SUMMARY - SAMENVATTING

The use of antibiotics as antimicrobial growth-promoters (AMGP) in pig feeds has been banned in the EU from January 2006 (regulation EC/1831/2003) and is under debate in other parts of the world. For decades the use of these AMGP in intensive pig production permitted higher growth rates and better feed conversion ratios in a nearly consistent way. Their mode of action is not fully understood but there is evidence that they work in part by decreasing the overall numbers and/or specific bacterial species or their metabolic activities in the gastro-intestinal tract (GIT). However, others believe that the growth-promoting effect can be attributed to a direct effect on host cells, in particular inflammatory cells. Furthermore, in the EU antimicrobial inclusion levels of copper in piglet feed is foreseen to be reduced in the future. In this respect, essential oils (EO) with well documented antimicrobial properties like carvacrol and *E*-cinnamaldehyde have gained renewed interest for use in animal feed. The specific objectives of the present study were: [1] to characterise the antimicrobial activities of EO against piglet intestinal bacteria; [2] to study the stability and kinetics of EO in the GIT of piglets and [3] to study the effect of EO on the functionality of the gut in piglets. Concerning the experimental work, effects were mainly quantified related to the weaning period of pigs, because in current pig production systems, the weaning period is one of the main critical points for growth and survival of the animal.

In Chapter 1, a literature review is presented. It comprises relevant data of carvacrol, thymol, anethole, eugenol and cinnamaldehyde, with special emphasis on the antimicrobial activities and the reported effects on pig gut bacteria and physiology and animal performance.

The antimicrobial activity of 7 EO against the major culturable components of the pig gut flora has been characterised by means of an *in vitro* incubation model simulating the fermentation in different

sections of the pig GIT (Chapter 2). In a first study, these compounds were screened for their antimicrobial properties. Dose-response equations were established for the 4 compounds with the highest potential in a second study. Binary combinations were tested as well, and the interaction effects were evaluated following the isobole method. The results of both studies indicated that carvacrol, thymol, eugenol and *E*-cinnamaldehyde give opportunities to modulate the flora and fermentation pattern of the GIT of pigs, while eucalyptol, terpinen-4-ol and *E*-anethole were found not to have interesting effects on modifying the composition of the pig gut flora. The minimum concentration for carvacrol, thymol, eugenol and *E*-cinnamaldehyde in jejunal simulations needed to reduce the number of total anaerobic bacteria compared to control with a probability of 99.7% was 255, 258, 223 and 56 mg/L respectively. This strong activity of *E*-cinnamaldehyde was due to its progressively increasing effect against coliform bacteria; a dose of 104 mg/L gave a reduction of 1 $\log_{10}$ CFU/mL vs. 371, 400 and 565 mg/L for carvacrol, thymol and eugenol respectively. However, *E*-cinnamaldehyde showed clearly less inhibitory activity towards lactobacilli than carvacrol and thymol. Therefore, the use of *E*-cinnamaldehyde (for example 100 mg/L) and to a lesser extent eugenol could result in a shift in the microbial ecology in favour of lactic acid producing bacteria and reducing the number of coliform bacteria. Carvacrol and thymol showed very similar and non-selective antimicrobial properties. Their effect was more pronounced in acidic media and demonstrated a rapidly increasing bactericidal effect from a certain concentration on (400-500 mg/L in jejunal simulations). The inhibition of the production of total SCFA in jejunal simulations by these 4 candidates was related to their effect against coliform bacteria, however they did not alter the lactic acid and ammonia concentrations. Few combinations demonstrated synergism; most mixtures showed zero interaction or antagonism. Carvacrol + thymol (ratio $\geq$1) was synergistic against total anaerobic bacteria in jejunal simulations, however this effect was rather small. In caecal simulations, carvacrol, thymol and *E*-cinnamaldehyde were equally effective while eugenol had only an effect on coliforms.

Data on the stability and kinetics of carvacrol, thymol, eugenol and *E*-cinnamaldehyde in the GIT of pigs were determined in a subsequent study (Chapter 3). Samples of *in vitro* incubations were analysed for their EO content, and the recovery was calculated as a measure for stability/degradation. None of these compounds was significantly degraded in *in vitro* simulations of the pig gastric fermentation. Carvacrol and thymol were not degraded in jejunal simulations, but significant losses up to 29% were found in caecal simulations. Eugenol and *E*-cinnamaldehyde showed a more pronounced degradation in jejunal and caecal simulations. A single dose mixed with feed (13.0, 13.2, 12.5 and 12.7 mg/kg BW for carvacrol, thymol, eugenol and *E*-cinnamaldehyde respectively) was given orally to piglets. Half-lives in total digestive tract ranged between 1.84 and 2.05 h, whereby *E*-cinnamaldehyde showed the fastest disappearance. All of these EO were mainly and nearly completely absorbed in the stomach and the proximal SI. Plasma concentrations (sum of free and conjugated compound) peaked at 1.39, 1.35 and 0.83 h for carvacrol, thymol and eugenol respectively and this was accompanied by high concentrations in urine.

The objectives in a choice-feeding study were: [1] to determine the effect of dose of thymol on palatability (experiment I) and [2] to study the possibility of added flavours to mitigate the aversive effects of high thymol inclusion rates on palatability (experiment II) (Chapter 4A). Twenty-four fully weaned piglets were divided into 8 groups of 3 animals. Each group was housed in a separated pen equipped with two troughs; a reference diet was offered in one trough and a test diet in the second trough. Piglets had free access to both troughs and could therefore choose between the two diets offered. Feed intake of the test diet was expressed as the proportion of the total intake and tested by means of a one-sample Student's t-test against a set value of 50% (n=16). The preference of the piglets for feed supplemented with 125, 500 and 2000 mg/kg thymol was 53.0 ± 14.0 % ($P>0.05$), 47.5 ± 19.6 % ($P>0.05$) and 9.9 ± 9.2 % ($P<0.001$) respectively (experiment I). Definitively, 125 and 500 mg/kg thymol did not affect palatability, while the piglets almost completely refused to eat feed with 2000 mg/kg thymol. Two test diets were included in experiment II; feed supplemented with

2000 mg/kg thymol with either flavour A (intended to mask the pungent taste of thymol) or flavour B (designed to mask both the taste and smell of thymol) and opposed against a control diet. The relative intake of test diets was 19.5 ± 10.5 % (P<0.001) and 14.0 ± 15.7 % (P<0.001), respectively, which means that the flavours could partially alleviate the aversive effects of 2000 mg/kg thymol. Alternatively, animals were offered one of these test diets and a reference diet containing 2000 mg/kg thymol. In accordance to the former results, animals had a preference for the feed with 2000 mg/kg thymol + flavour A (67.6 ± 18.9 %; P<0.01). However, the results for the feed with 2000 mg/kg thymol + flavour B (28.9 ± 13.1 %; P<0.001) did not confirm the previous observations.

Two trials were conducted to study the effect of dose and formulation of carvacrol and thymol on gut bacterial counts and metabolites and gut function in weaned piglets. In experiment I, 25 piglets (28 d, 6.59 ± 0.48 kg) were allocated to five dietary treatments: a control diet and the same diet supplemented with either carvacrol or thymol at doses of 500 and 2000 mg/kg. The EO were dissolved in the soybean oil fraction, prior to mixing with the basal diet. In experiment II, 35 piglets (28 d, 7.99 ± 0.73 kg) were assigned to seven dietary treatments: the same control diet as in experiment I and this diet supplemented with thymol in one of three formulations (on celite, on alphacel or microencapsulated) at doses of 500 and 2000 mg/kg. Microencapsulation was applied in order to delay the release of the active ingredient in the gastro-intestinal tract. All diets were given *ad libitum*. At 11/12 days post-weaning piglets were euthanized, and digesta from stomach, proximal and distal SI were sampled for bacteriological (plating onto selective media for total anaerobic bacteria, coliforms, streptococci and lactobacilli) and biochemical analysis. Small intestinal tissue was sampled for histo-morphological determinations. Feed intake was higher for animals fed 500 mg/kg carvacrol and thymol, with the exception of the microencapsulation treatment (between +12.4 and +44.7% as compared to control), and likewise villi at the distal SI were larger compared to control (between +4.9 and +7.5% as compared to control; P>0.05). In experiment I, the villus/crypt ratio at the distal SI for the experimental diets (1.30-1.32) was higher than for the control diet (1.24)

(P<0.05). Thymol fed animals in experiment II had a lower number of intra-epithelial lymphocytes at the proximal (P<0.05) and at the distal (P<0.1) SI as compared to the control animals. Concurrently, the villus/crypt ratio at the distal SI tended to be higher in the experimental diets (P<0.1). Mean EO concentration in stomach and proximal SI for the 2000 mg/kg diets ranged between 475 and 647 and between 5 and 24 mg/kg fresh digesta, respectively. Cumulative absorption in proximal SI was higher than 90% for all treatments. These data suggest that EO can enhance feed intake at 500 mg/kg and improve gut health, but conclusive evidence for clear antimicrobial effects towards the major culturable bacteria of the pig gut is limited.

Chapter 5 presents a detailed study of the effects of thymol, *E*-anethole and *E*-cinnamaldehyde on active epithelial absorption of D-glucose and L-alanine and induced chloride secretion in the Ussing chamber model. Stripped mid-jejunal tissue of fully weaned piglets was mounted in modified Ussing chambers. The increase in short-circuit current (ΔIsc) to mucosal addition of 16 mmol/L D-glucose and 16 mmol/L L-alanine, with or without pre-incubation with 0.2 and 1.0 mmol/L thymol or *E*-cinnamaldehyde and 0.2 and 0.5 mmol/L *E*-anethole was determined. Accordingly, the effect on stimulated chloride secretion by secretagogues was measured. Thymol and *E*-cinnamaldehyde reduced the Isc response to nutrients and secretagogues in a dose-dependent manner. ΔIsc to D-glucose for thymol at 0.2 and 1.0 mmol/L was respectively 73.7 ± 7.9 (P>0.05) and 23.8 ± 7.4 (P<0.001) compared to 80.1 ± 7.4 μA/cm^2 for control and to L-alanine respectively 20.6 ± 2.3 (P=0.01) and 5.9 ± 2.4 (P<0.001) compared to 33.7 ± 2.6 μA/cm^2 for control. *E*-cinnamaldehyde showed only significant effects at 1 mmol/L. Such effects *in vivo* could compromise intestinal absorption of nutrients. The reduction in secretory response to cAMP/cGMP mediated VIP and theophylline was greater than for Ca^{2+} mediated 5-HT and carbachol. Pre-incubation with 0.5 mmol/L *E*-anethole did not alter response to mucosal D-glucose, but affected chloride secretion induced by 5-HT and theophylline. This could imply that the EO would be beneficial in cases of excessive chloride secretion that results in the clinical picture of diarrhoea, *e.g.* in response to toxins

such as the cholera toxin or ETEC enterotoxins whose mechanisms of action involve the neurocrine mediators.

Finally, a general discussion (Chapter 6) aims at interrelating all data. Data are viewed in the perspective of these EO as alternatives for the AMGP and future prospects are discussed. To conclude, the *in vitro* studies showed the huge potential of carvacrol, thymol and *E*-cinnamaldehyde to modulate the composition of the gut bacteria of the pig. Contrary, in two trials with weaned piglets the supplementation with carvacrol and thymol at the doses applied did not reduce the bacterial load, most likely due to fast absorption of the active ingredient in the stomach and the proximal small intestine. Some effects indicate that the gut health balance might be improved, however the Ussing chamber study showed deleterious effects on active nutrient absorption. It is argued that there is still scope to improve the efficacy of these EO to aid the development and function of the GIT in piglets.

Het gebruik van antibiotica als antimicrobiële groeibevorderaar (AMGB) in de voeding van varkens is sinds 2006 in de EU verboden (Verordening EC/1831/2003) en staat ter discussie in de rest van de wereld. De toepassing van deze AMGB's in de intensieve varkensproductie gedurende de laatste decennia gaf aanleiding tot een betere groei van de dieren en een verlaagde voederconversie. De werkingswijze waarbij dit gebeurde is niet volledig opgehelderd maar er is genoeg wetenschappelijk bewijs om aan te nemen dat het gunstig effect toe te schrijven is aan een remmende werking op de bacteriën en hun metabolisme in het maag-darmkanaal. Alhoewel andere auteurs het groeibevorderend effect van de AMGB's eerder in verband brengen met een direct effect op de cellen van de gastheer, in het bijzonder de ontstekingscellen. Verder wordt verwacht dat de toegelaten antimicrobiële dosis van koper in de biggenvoeding in de EU in de toekomst zal teruggebracht worden. Omdat van sommige essentiële oliën (EO), zoals de componenten carvacrol en kaneelaldehyde bekend is dat ze antimicrobiële eigenschappen bezitten, is er aldus interesse om EO te gebruiken in diervoeders. De specifieke doelstellingen van het huidige proefschrift waren: [1] de antimicrobiële eigenschappen van deze EO tegenover de darmbacteriën van het varken te karakteriseren, [2] de stabiliteit en de kinetiek van de EO in het maag-darmkanaal te onderzoeken en [3] het effect van deze componenten op de functionaliteit van de darm bij biggen te bepalen. In deze studie werden de effecten nagegaan bij gespeende biggen, omdat in de huidige productiesystemen, de speenfase één van de meest kritieke fasen is voor de groei en overleving van het dier.

Hoofdstuk 1 bevat een overzicht van de bestaande literatuur. Het behandelt relevante gegevens met betrekking tot carvacrol, thymol, anethol, eugenol en kaneelaldehyde, met bijzondere aandacht voor hun antimicrobiële eigenschappen en waargenomen effecten op de bacteriën van het maag-darmkanaal, de darmfysiologie en de prestaties van biggen.

De antimicrobiële activiteit ten aanzien van de belangrijkste cultiveerbare groepen darmbacteriën bij het varken van 7 EO werd gekarakteriseerd door middel van *in vitro* simulaties van de fermentatie in

de verschillende delen van het maag-darmkanaal (Hoofdstuk 2). In een eerste studie werd een screening uitgevoerd. Daarna werden dosis-respons relaties opgesteld van de 4 componenten met het grootste potentieel. Binaire combinaties werden eveneens getest, en de interactie werd geëvalueerd door middel van de isobool methode. De resultaten van beide studies toonden aan dat carvacrol, thymol, eugenol en *E*-kaneelaldehyde mogelijkheden bieden om de groei van de bacteriën en het fermentatiepatroon in het maag-darmkanaal van varkens te moduleren. Eucalyptol, terpinen-4-ol en *E*-anethol vertoonden geen interessante effecten. De minimale concentratie van carvacrol, thymol, eugenol en *E*-kaneelaldehyde nodig om de aantallen bacteriën in simulaties van de dunne darm fermentatie te reduceren met een probabiliteit van 99.7% was respectievelijk 255, 258, 223 and 56 mg/L. *E*-kaneelaldehyde vertoonde een uitgesproken activiteit ten aanzien van coliforme bacteriën, een dosis van 104 mg/L gaf een reductie van 1 $\log_{10}$ KVE (kolonievormende eenheden)/mL vs. 371, 400 en 565 mg/L voor respectievelijk carvacrol, thymol en eugenol. Daartegenover staat dat *E*-kaneelaldehyde minder effectief was tegen lactobacillen dan carvacrol en thymol. De toepassing van *E*-kaneelaldehyde en in mindere mate eugenol kan dus resulteren in een verschuiving van de microbiële ecologie ten gunste van melkzuurproducerende bacteriën en ten nadele van coliformen. Carvacrol en thymol hadden vergelijkbare en niet-selectieve antimicrobiële eigenschappen. Hun effect was het meest uitgesproken in een zuur medium en ze vertoonden een sterk toenemend bactericidaal effect eens een bepaalde kritieke concentratie (400-500 mg/L in simulaties van dunne darm) werd overschreden. De reductie door de 4 componenten van de kort-keten vetzuurproductie was gerelateerd aan het effect ten aanzien van de coliforme bacteriën, ze hadden geen effect op de melkzuur en ammonium concentraties in het medium. Een beperkt aantal combinaties was synergetisch, de meeste mengsels resulteerden in geen interactie of antagonisme. Carvacrol + thymol (ratio $\geq$1) vertoonde synergisme tegen totaal anaërobe bacteriën in simulaties van de dunne darm, maar het effect was beperkt. In caecum simulaties waren carvacrol, thymol en *E*-kaneelaldehyde even effectief, terwijl eugenol enkel een effect op de coliformen had.

Gegevens met betrekking tot de stabiliteit en de kinetiek in het maag-darmkanaal van carvacrol, thymol, eugenol en *E*-kaneelaldehyde werden bepaald in een volgende studie (Hoofstuk 3). De concentratie van de EO in het medium van *in vitro* incubaties werd gekwantificeerd, en de terugwinst na incubatie werd berekend als een maat voor stabiliteit/degradatie. Geen enkele van deze componenten werd significant afgebroken in *in vitro* simulaties van de maag fermentatie. Voor carvacrol en thymol werden evenmin significante verliezen in dunne darm simulaties vastgesteld, maar wel in caecum simulaties (tot 29%). Belangrijke degradatie van eugenol en *E*-kaneelaldehyde trad op in dunne darm en caecum simulaties. Aan biggen werd oraal één enkele dosis van carvacrol, thymol, eugenol en *E*-kaneelaldehyde gemengd met het voeder (respectievelijk 13.0, 13.2, 12.5 en 12.7 mg/kg lichaamsgewicht) toegediend. Halfwaardetijden voor het totale maag-darmkanaal lagen tussen 1.84 en 2.05 u, waarbij de snelste verdwijning optrad voor *E*-cinnamaldehdye. Al deze stoffen werden snel en nagenoeg volledig geabsorbeerd in de maag en het duodenum, met als gevolg dat lage concentraties werden aangetroffen in de meer distale delen. Plasma concentraties (som van de vrije en geconjugeerde component) piekte na 1.39, 1.35 en 0.85 u voor respectievelijk carvacrol, thymol en eugenol en dit ging gepaard met hoge concentraties in de urine.

De doelstellingen in een choice-feeding studie waren: [1] het bepalen van het effect van de dosis van thymol op de smakelijkheid (experiment I) en [2] het bestuderen van de mogelijkheid van toegevoegde smaakstoffen om de negatieve effecten van een hoge dosis thymol te maskeren (experiment II) (Hoofdstuk 4A). Vierentwintig volledige gespeende biggen werden verdeeld in 8 groepen van 3 dieren. Elke groep werd gehuisvest in een afzonderlijk hok dat voorzien was van twee identieke voederbakken. Een referentie voeder werd verstrekt in de ene voederbak en een test voeder in de andere voederbak. De dieren hadden toegang tot beide voederbakken en konden dus vrij kiezen tussen de twee voeders. De voederopname van het testvoeder werd uitgedrukt als de proportie van de totale opname. Door middel van een one-sample Student's t-test werd nagegaan of deze relatieve opname statistisch verschillend was van 50% (n=16). De voorkeur van de biggen voor het voeder met

125, 500 en 2000 mg/kg thymol was 53.0 ± 14.0 % (P>0.05), 47.5 ± 19.6 % (P>0.05) en 9.9 ± 9.2 % (P<0.001) respectievelijk (experiment I). Het betekent dat 125 en 500 mg thymol per kg voeder geen effect had op de smakelijkheid, terwijl de biggen haast volledig het voeder met 2000 mg/kg thymol links lieten liggen. In experiment II werden twee testvoeders gebruikt; een voeder dat gesupplementeerd was met 2000 mg/kg thymol met ofwel smaakstof A (bedoeld om de smaak van thymol te maskeren) of met smaaktstof B (om smaak en geur van thymol te maskeren). Deze werden getest tegenover een controle voeder. De relatieve opname van het testvoeder was respectievelijk 19.5 ± 10.5 % (P<0.001) en 14.0 ± 15.7 % (P<0.001), wat erop duidt dat de smaakstoffen de afkeer ten aanzien van 2000 mg/kg thymol gedeeltelijk konden reduceren. Vervolgens werden de dieren gevoederd met dezelfde testvoeders, maar als referentievoeder werd hier een voeder met 2000 mg/kg thymol verstrekt. In overeenstemming met de vorige resultaten, verkozen de dieren het voeder met 2000 mg/kg thymol + smaakstof A (67.6 ± 18.9 %; P<0.01). Het resultaat voor 2000 mg/kg thymol + smaaktstof B (28.9 ± 13.1 %; P<0.001) bevestigde evenwel niet de vorige bevindingen.

In twee proeven met gespeende biggen werden de voeders gesupplementeerd met carvacrol en thymol, en dit bij dosissen van 500 en 2000 mg/kg voeder (Hoofdstuk 4B). Het effect op de aantallen darmbacteriën en metabolieten en de darmfunctie werd onderzocht. In experiment I werden 25 gespeende biggen (28 d, 6.59 ± 0.48 kg) verdeeld over 5 voederbehandelingen: een controle voeder en hetzelfde voeder waaraan carvacrol of thymol hetzij aan een dosis van 500 of 2000 mg/kg werd toegevoegd. De EO werden opgelost in de sojaolie, vooraleer het werd gemengd met de rest van het voeder. In experiment II werden 35 gespeende biggen (28 d, 7.99 ± 0.73 kg) verdeeld over 7 behandelingen: een controle voeder en hetzelfde voeder gesupplementeerd met thymol in één van drie formulaties (op celite, op alphacel of na microencapsulatie) en dit bij 500 en 2000 mg/kg. Microencapsulatie werd toegepast met als bedoeling de vrijstelling van de actieve component in het maag-darmkanaal te vertragen. De voeders werden *ad libitum* verstrekt. Na 11/12 dagen werden de dieren geëuthanaseerd en de digesta van de maag, de proximale en distale dunne darm werden

bemonsterd voor bacteriologische (uitplaten op selectieve media voor totaal anäerobe bacteriën, coliformen, streptococcen en lactobacillen) en biochemische analyses. Stalen van dunne darmweefsel werden genomen voor histo-morfologische bepalingen. De voederopname was hoger bij dieren gevoederd met 500 mg/kg carvacrol en thymol, met uitzondering van de microencapsulatie behandeling (tussen +12.4 en +44.7% in vergelijking met controle), en bijgevolg waren de darmvilli ter hoogte van de distale dunne darm ook groter dan bij de controle dieren (tussen +4.9 en +7.5%; $P>0.05$). In experiment I was de verhouding villushoogte/cryptediepte ter hoogte van distale dunne darm hoger voor de experimentele voeders (1.30-1.32) dan voor het controle voeder (1.24) ($P<0.05$). Dieren gevoederd met thymol in experiment II vertoonden een lager aantal intra-epitheliale lymfocyten in de proximale ($P<0.05$) en de distale ($P<0.1$) dunne darm in vergelijking met controle dieren. Deze laatste bevinding kan in verband gebracht worden met de tendens tot een verhoogde verhouding villushoogte/cryptediepte ter hoogte van distale dunne darm bij de experimentele voeders ($P<0.1$). De gemiddelde concentratie van de EO in de verse digesta van maag en proximale dunne darm voor de behandelingen met 2000 mg/kg thymol varieerde tussen 475 en 647 en tussen 5 en 24 mg/kg respectievelijk. De gecumuleerde absorptie in de proximale dunne darm was voor alle behandelingen hoger dan 90%. De resultaten uit deze proeven suggeren dat deze EO de voederopname kunnen verhogen bij 500 mg/kg en daarnaast de darmgezondheid positief beïnvloeden, maar duidelijke antimicrobiële effecten tegenover de belangrijkste cultiveerbare darmbacteriën werden niet gevonden.

In het Ussing kamer model werd het effect van thymol, *E*-anethol en *E*-kaneelaldehyde op actieve nutriënt absorptie en secretie nagegaan (Hoofdstuk 5). Gestript weefsel afkomstig van mid-jejunum van volledig gespeende biggen werd gemonteeerd in gemodificeerde Ussing kamers. De stijging in de stroom bij kortsluiten (ΔIsc, short-ciruit current) na mucosale additie van 16 mmol/L D-glucose en 16 mmol/L L-alanine, al of niet na pre-incubatie met 0.2 en 1.0 mmol/L thymol of *E*-kaneelaldehyde en 0.2 en 0.5 mmol/L *E*-anethol werd onderzocht. Op dezelde manier werd het effect op

gestimuleerde chloride secretie door middel van secretagogen opgemeten. Thymol en *E*-kaneelaldehyde reduceerden op een dosis afhankelijke wijze de absorptie van D-glucose en L-alanine en gestimuleerde secretie van chloride. ΔIsc voor D-glucose met thymol aan 0.2 en 1.0 mmol/L was respectievelijk 73.7 ± 7.9 (P>0.05) en 23.8 ± 7.4 (P<0.001) in vergelijking met 80.1 ± 7.4 μA/cm^2 voor de controle en voor L-alanine respectievelijk 20.6 ± 2.3 (P=0.01) en 5.9 ± 2.4 (P<0.001) tegenover 33.7 ± 2.6 μA/cm^2 voor de controle. Enkel bij 1 mmol/L vertoonde *E*-kaneelaldehyde significante effecten. Indien dergelijke effecten zich *in vivo* zouden voordoen, kan dit de actieve absorptie van nutriënten in gevaar brengen. De reductie van de geïnduceerde chloride secretie via cAMP/cGMP gemedieerde VIP en theophylline was groter dan voor Ca^{2+} gemedieerde 5-HT en carbachol. Pre-incubatie met 0.5 mmol/L *E*-anethol gaf geen effect op de de Isc respons na mucosale additie van D-glucose, maar wel op chloride secretie door 5-HT en theophylline. Het feit dat deze EO de respons op secretagogen reduceren kan positief zijn in gevallen van overmatige chloride secretie dat aanleiding geeft tot klinische diarree; bijvoorbeeld in respons tot toxines afkomstig van enterotoxische *E. coli*. Echter, in dit onderzoek werden ook aanwijzingen gevonden die erop kunnen wijzen dat cytotoxische effecten een rol hebben gespeeld.

In het laatste hoofdstuk worden de bekomen resultaten verder bediscussieerd en geïnterpreteerd en dit in licht van deze EO als alternatief voor de AMGB's. Als conclusie kan gesteld worden dat het *in vitro* onderzoek het enorme potentieel van carvacrol, thymol en kaneelaldehyde om de samenstelling van de darmbacteriën bij het varken te moduleren heeft aangetoond. Echter, in twee proeven met gespeende biggen werd vastgesteld dat de supplementatie met carvacrol en thymol, bij de dosissen die werden toegepast geen reductie van de aantallen bacteriën opleverde. Dit was hoogst waarschijnlijk te wijten aan de snelle absorptie van de actieve component in de maag en de proximale dunne darm. Sommige effecten duidden op een verbeterde darmgezondheidheidstoestand, maar de Ussing kamer studie toonde aan dat deze stoffen een nefast effect hadden op de actieve absorptie van

nutriënten. Niettemin wordt aangetoond dat er perspectieven zijn om de effectiviteit van deze componenten te verbeteren.

REFERENCES

Abid, A., Bouchon, I., Siest, G. & Sabolovic, N. (1995). Glucuronidation in the caco-2 human intestinal cell line: induction of UDP-glucuronosyltransferase 1*6. Biochemical Pharmacology, **50** (4), 557-561.

Ader, P., Blöck, M., Pietzsch, S. & Wolffram, S. (2001). Interaction of quercetin glucosides with the intestinal sodium/glucose co-transporter (SGLT-1). Cancer Letters, **162** (2), 175-180.

Aeschbach, R., Löliger, J., Scott, B. C., Murcia, A., Butler, J., Halliwell, B. & Aruoma, O. I. (1994). Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food and Chemical Toxicology, **32** (1), 31-36.

Albin, K. C., Carstens, M. I. & Carstens, E. (2008). Modulation of oral heat and cold pain by irritant chemicals. Chemical Senses, **33** (1), 3-15.

Ali, S. M., Khan, A. A., Ahmed, I., Musaddiq, M., Ahmed, K. S., Polasa, H., Venkateshwar Rao, L., Habibullah, C. M., Sechi, L. A. & Ahmed, N. (2005). Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. Annals of Clinical Microbiology and Antimicrobials, **4** (1), 20.

Aligiannis, N., Kalpoutzakis, E., Mitatku, S. & Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two *Origanum* species. Journal of Agricultural and Food Chemistry, **49**, 4168-4170.

Allan, P. & Bilkei, G. (2005). Oregano improves reproductive performance of sows. Theriogenology, **63** (3), 716-721.

Amrik, B. & Bilkei, G. (2004). Influence of farm application of oregano on performance of sows. Canadian Veterinary Journal, **45**, 674-677.

Andersen, A. (2006). Final report on the safety assessment of sodium *p*-chloro-*m*-cresol, *p*-chloro-*m*-cresol, chlorothymol, mixed cresols, *m*-cresol, *o*-cresol, *p*-cresol, isopropyl cresols, thymol, *o*-cymen-5-ol, and carvacrol. International Journal of Toxicology, **25**, 29-127.

Anderson, D. B., McCracken, V. J., Aminov, R. I., Simpson, J. M., Mackie, R. I., Verstegen, M. W. A. & Gaskins, H. R. (1999). Gut microbiology and growth-promoting antibiotics in swine. Pig News and Information, **20** (4), 115N-122N.

Anderson, R. A., Broadhurst, C. L., Polansky, M. M., Schmidt, W. F., Khan, A., Flanagan, V. P., Schoene, N. W. & Graves, D. J. (2004). Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. Journal of Agricultural and Food Chemistry, **52** (1), 65-70.

Andersson, D. A., Gentry, C., Moss, S. & Bevan, S. (2008). Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. The Journal of Neuroscience, **28** (10), 2485-2494.

Andrés Elias, N., Badiola, I., Pujols, J. & Torrallardona, D. (2007). Effects of cinnamaldehyde, benzoic acid and grapefruit extract on gut health of weanling pigs. In: Book of Abstracts of the 58th Annual Meeting of EAAP, 26-30 August 2007 Dublin, Ireland.

Anonymous (1996). Thymol. Summary report by the Committee for Veterinary Medicinal Products (EMEA/MRL/075/96-FINAL). The European Agency for the Evaluation of Medicinal Products.

Anonymous (2000). Thymol No. 259, Cas No.89-83-8. Toxicological Evaluation. Berufsgenossenschaft der chemischen Industrie, Heidelberg, Germany. www.bgchemie.de/toxicologicalevaluations, 35.

Anonymous (2004). Evaluation of certain food additives and contaminants: sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives (WHO technical report series ; 922), Geneva, 2004

Apajalahti, J. & Kettunen, A. (2006). Rational development of novel microbial modulators. In: Barug, D., De Jong, J., Kies, A.K., Verstegen, M.W.A. (Eds), Antimicrobial growth-promoters. Where do we go from here? Wageningen Academic Publishers, Wageningen, pp.165-181.

Aromatic Consortium (2005). Revised test plan for cinnamyl derivatives. Report nr. 201-15843A, submitted to the EPA under the HPV Challenge Program by: The Flavor and Fragrance High Production Volume Chemical Consortia, The Aromatic Consortium, Washington, D.C. 20006, 41p.

Austgulen, L. T., Solheim, E. & Scheline, R. R. (1987). Metabolism in rats of para-cymene derivatives - carvacrol and thymol. Pharmacology & Toxicology, **61** (2), 98-102.

Aydin, Y., Kutlay, O., Ari, S., Duman, S., Uzuner, K. & Aydin, S. (2007). Hypotensive effects of carvacrol on the blood pressure of normotensive rats. Planta Medica, **73** (13), 1365-1371.

Baker, W. L., Gutierrez-Williams, G., White, C. M., Kluger, J. & Coleman, C. I. (2008). Effect of cinnamon on glucose control and lipid parameters. Diabetes Care, **31** (1), 41-43.

Bakkali, F., Averbeck, S., Averbeck, D. & Idaomar, M. (2008). Biological effects of essential oils - a review. Food and Chemical Toxicology, **46** (2), 446-475.

Bandell, M., Story, G. M., Hwang, S. W., Viswanath, V., Eid, S. R., Petrus, M. J., Earley, T. J. & Patapoutian, A. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron, **41** (6), 849-857.

Barrett, K. E. & Keely, S. J. (2000). Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. Annual Review of Physiology, **62**, 535-572.

Basketter, D. (2000). Quenching: fact or fiction? Contact Dermatitis, **43** (5), 253-258.

Bauermann, U. (2006). Dectetion please! Essential oils in animal nutrition. Feed Magazine / Kraftfutter, (4), 19-21.

Becerril, R., Gomez-Lus, R., Goni, P., Lopez, P. & Nerin, C. (2007). Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against *E. coli* and *S. aureus*. Analytical and Bioanalytical Chemistry, **388** (5-6), 1003-1011.

Beer, A. M., Lukanov, J. & Sagorchev, P. (2007). Effect of thymol on the spontaneous contractile activity of the smooth muscles. Phytomedicine, **14** (1), 65-69.

Beesley, A., Hardcastle, J., Hardcastle, P. T. & Taylor, C. J. (1996). Influence of peppermint oil on absorptive and secretory processes in rat small intestine. Gut, **39** (2), 214-219.

Ben Arfa, A., Chrakabandhu, Y., Preziosi-Belloy, L., Chalier, P. & Gontard, N. (2007). Coating papers with soy protein isolates as inclusion matrix of carvacrol. Food Research International, **40** (1), 22-32.

Benchaar, C., Calsamiglia, S., Chaves, A. V., Fraser, G. R., Colombatto, D., McAllister, T. A. & Beauchemin, K. A. (2008). A review of plant-derived essential oils in ruminant nutrition and production. Animal Feed Science and Technology, **145** (1-4), 209-228.

Berenbaum, M.C. (1985). The expected effect of a combination of agents: the general solution. Journal of Theoretical Biology, **114**, 413-431.

Berenbaum, M.C. (1989). What is synergy? Pharmacological Reviews, **41**, 93-141.

Bergonzelli, G. E., Donnicola, D., Porta, N. & Corthésy-Theulaz, I. E. (2003). Essential oils as components of a diet-based approach to management of *Heliobacter* infection. Antimicrobial Agents and Chemotherapy, **47** (10), 3240-3246.

Bevilacqua, A., Corbo, M. R. & Sinigaglia, M. (2008). Combined effects of low pH and cinnamaldehyde on the inhibition of *Alicyclobacillus acidoterrestris* spores in a laboratory medium. Journal of Food Processing and Preservation, **32** (5), 839-852.

Bhattaram, V. A., Graefe, U., Kohlert, C., Veit, M. & Derendorf, H. (2002). Pharmacokinetics and bioavailability of herbal medicinal products. Phytomedicine, **9**, Suppl. 3, 1-33.

Bickers, D., Calow, P., Greim, H., Hanifin, J. M., Rogers, A. E., Saurat, J. H., Sipes, I. G., Smith, R. L. & Tagami, H. (2005). A toxicologic and dermatologic assessment of cinnamyl alcohol, cinnamaldehyde and cinnamic acid when used as fragrance ingredients. Food and Chemical Toxicology, **43**, 799-836.

Bimczok, D., Rau, H., Sewekow, E., Janczyk, P., Souffrant, W. B. & Rothkotter, H. J. (2008). Influence of carvacrol on proliferation and survival of porcine lymphocytes and intestinal epithelial cells *in vitro*. Toxicology in Vitro, **22** (3), 652-658.

Blaszyk, M. & Holley, R. A. (1998). Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. International Journal of Food Microbiology, **39** (3), 175-183.

Bock, G., Benda, I. & Schreier, P. (1988). Reduction of cinnamaldehyde and unsaturated acids by *Botrytis cinerea* Zeitschrift für Lebensmittel-Untersuchung und Forschung, **186**, 33-35.

Boskabady, M. H. & Jandagh, P. (2003). Relaxant effect of carvacrol on guinea pig tracheal chains and its possible mechanisms. Pharmazie, **58**, 661-663.

Boudry, G. & Perrier, C. (2008). Thyme and cinnamon extracts induce anion secretion in piglet small intestine via cholinergic pathways. Journal of Physiology and Pharmacology, **59** (3), 543-552.

Boudry, G. (2005). The Ussing chamber technique to evaluate alternatives to in-feed antibiotics for young pigs Animal Research, **54**, 219-230.

Bounds, S. V. & Caldwell, J. (1996). Pathways of metabolism of [1'-C-14]-*trans*-anethole in the rat and mouse. Drug Metabolism and Disposition, **24** (7), 717-724.

Boyland, E. & Chasseaud, L. F. (1970). The effect of some carbonyl compounds on rat liver glutathione levels. Biochemical Pharmacology, **19** (4), 1526-1528.

Brackman, G., Defoirdt, T., Miyamoto, C., Bossier, P., Van Calenbergh, S., Nelis, H. & Coenye, T. (2008). Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. BMC Microbiology, **8**: 149.

Braga, P. C., Dal Sasso, M., Culici, M., Bianchi, T., Bordoni, L. & Marabini, L. (2006). Anti-inflammatory activity of thymol: inhibitory effect on the release of human neutrophil elastase. Pharmacology, **77** (3), 130-136.

Broudiscou, L. P., Cornu, A. & Rouzeau, A. (2007). *In vitro* degradation of 10 mono- and sesquiterpenes of plant origin by caprine rumen micro-organisms. Journal of the Science of Food and Agriculture, **87** (9), 1653-1658.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology, **94**, 223-253.

Burt, S. A. & Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology, **36**, 162-167.

Burt, S. A., Fledderman, M. J., Haagsman, H. P., van Knapen, F. & Veldhuizen, E. J. A. (2007a). Inhibition of *Salmonella enterica* serotype Enteritidis on agar and raw chicken by carvacrol vapour. International Journal of Food Microbiology, **119** (3), 346-350.

Burt, S. A., van der Zee, R., Koets, A. P., de Graaff, A. M., van Knapen, F., Gaastra, W., Haagsman, H. P. & Veldhuizen, E. J. A. (2007b). Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7. Applied and Environmental Microbiology, **73** (14), 4484-4490.

Burt, S. A., Vlielander, R., Haagsman, H. P. & Veldhuizen, E. J. A. (2005). Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers. Journal of Food Protection, **68** (5), 919-926.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L. & Ferret, A. (2007). Invited review: essential oils as modifiers of rumen microbial fermentation. Journal of Dairy Science, **90** (6), 2580-2595.

Campbell, N. B., Ruaux, C. G., Shifflett, D. E., Steiner, E. M., Williams, D. A., Blikslager, A. T. (2004). Physiological concentrations of bile salts inhibit recovery of ischemic-injured porcine ileum. American Journal of Physiology – Gastrointestinal and Liver Physiology, **287** (2), G399-G407.

Canibe, N. & Jensen, B. B. (2003). Fermented and nonfermented liquid feed to growing pigs: effect on aspects of gastrointestinal ecology and growth performance. Journal of Animal Science, **81**, 2019-2031.

Cardozo, P. W., Calsamiglia, S., Ferret, A. & Kamel, C. (2004). Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. Journal of Animal Science, **82**, 3230-3236.

Carlson, D., Poulsen, H. D. & Sehested, J. (2004). Influence of weaning and effect of post weaning dietary zinc and copper on electrophysiological response to glucose, theophylline and 5-HT in piglet small intestinal mucosa. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, **137** (4), 757-765.

Carlson, D., Sehested, J. & Poulsen, H. D. (2006). Zinc reduces the electrophysiological responses *in vitro* to basolateral receptor mediated secretagogues in piglet small intestinal epithelium. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, **144** (4), 514-519.

Casewell, M., Friis, C., Marco, E., McMullin, P. & Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy, **52** (2), 159-161.

Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F. & Gasa, J. (2006). The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. Journal of Animal Science, **84** (10), 2725-2734.

Cervenka, L., Peskova, I., Pejchalova, M. & Vytrasova, J. (2008). Inhibition of *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* by plant oil aromatics. Journal of Food Protection, **71** (1), 165-169.

Chainy, G. B. N., Manna, S. K., Chaturvedi, M. M. & Aggarwal, B. B. (2000). Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-κB, AP-1, JNK, MAPKK and apoptosis. Oncogene, **19** (25), 2943.

Chamberlain, E. M. & Dagley, S. (1968). The metabolism of thymol by a *Pseudomonas*. Biochemical Journal, **110**, 755-763.

Chan, A. S. L., Pang, H., Yip, E. C. H., Tam, Y. K. & Wong, Y. H. (2005). Carvacrol and eugenol differentially stimulate intracellular Ca^{2+} mobilization and mitogen-activated protein kinases in Jurkat T-cells and monocytic THP-1 cells. Planta Medica, **71**, 634-639.

Chao, L. K., Hua, K. F., Hsu, H. Y., Cheng, S. S., Lin, I. F., Chen, C. J., Chen, S. T. & Chang, S. T. (2008). Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. Food and Chemical Toxicology, **46** (1), 220-231.

Chao, S. C., Young, D. G. & Oberg, C. J. (2000). Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. Journal of Essential Oil Research, **12** (5), 639-649.

Chaves, A. V., Stanford, K., Gibson, L. L., McAllister, T. A. & Benchaar, C. (2008). Effects of carvacrol and cinnamaldehyde on intake, rumen fermentation, growth performance, and carcass characteristics of growing lambs. Animal Feed Science and Technology, **145** (1-4), 396-408.

Chemat, F., Abert Vian, M. & Dangles, O. (2007). Essential oils as antioxidants. International Journal of Essential Oil Therapeutics, **1**, 4-15.

Cheng, J.-T., Liu, I.-M., Huang, W.-C. & Kou, D. H. (2000). Stimulatory effect of *E-cinnamaldehyde* on noradrenaline secretion in guinea-pig ileum myenteric nerve terminals. Life Sciences, **66** (11), 981-990.

Chericoni, S., Prieto, J. M., Iacopini, P., Cioni, P. & Morelli, I. (2005). *In vitro* activity of the essential oil of cinnamomum zeylanicum and eugenol in peroxynitrite-induced oxidative processes. Journal of Agricultural and Food Chemistry, **53**, 4762-4765.

Close, W. H. (2000). Producing pig without antibiotic growth promoters. Advances in pork production, **11**, 47-56.

Cocchiara, J., Letizia, C. S., Lalko, J., Lapczynski, A. & Api, A. M. (2005). Fragrance material review on cinnamaldehyde. Food and Chemical Toxicology, **43**, 867-923.

Conway, E.J. (1962). Microdiffusion analysis and volumetric error. 5th Ed., Crosby Lockwood, London, 467p.

Corpet, D. E. (2000). Mécanismes de la promotion de croissance des animaux par les additifs alimentaires antibiotiques. Revue Médicins Vétérinaires, **151** (2), 99-104.

Cosentino, S., Tuberoso, C. I., Pisano, B., Satta, M., Mascia, V., Arzedi, E. & Palmas, F. (1999). *In-vitro* antimicrobial activity and chemical composition of Sardinian thymus essential oils. Letters of Applied Microbiology, **29** (2), 130-135.

Costa, L. B., Panhoza Tse, M. L. & Miyada, V. S. (2007). Herbal extracts as alternatives to antimicrobial growth promoters for weanling pigs. Brazilian Journal of Animal Science, **36** (3), 589-595.

Cowan, M. M. (1999). Plant products as antimicrobial agents. Clinical Microbiology Reviews, **12** (4), 564-582.

Cox, S. D. & Markham, J. L. (2007). Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. Journal of Applied Microbiology, **103** (4), 930-936.

Cox, S. D., Mann, C. M. & Markham, J. L. (2001). Interactions between components of the essential oil of *Melaleuca alternifolia*. Journal of Applied Microbiology, **91** (3), 492-497.

Cristani, M., D'Arrigo, M., Mandalari, G., Castelli, F., Sarpietro, M. G., Micieli, D., Venuti, V., Bisignano, G., Saija, A. & Trombetta, D. (2007). Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity. Journal of Agricultural and Food Chemistry, **55** (15), 6300-6308.

Cromwell, G. L. (2002). Why and how antibiotics are used in swine production. Animal Biotechnology, **13** (1), 7-27.

Cruz-Orengo, L., Dhaka, A., Heuermann, R. J., Young, T. J., Montana, M. C., Cavanaugh, E. J., Kim, D. & Story, G. M. (2008). Cutaneous nociception evoked by 15-delta PGJ2 via activation of ion channel TRPA1. Molecular Pain, **4**:30.

Dal Sasso, M., Culici, M., Braga, P. C., Guffanti, E. E. & Mucci, M. (2006). Thymol: inhibitory activity on *Escherichia coli* and *Staphylococcus aureus* adhesion to human vaginal cells. Journal of Essential Oil Research, **18** (4), 455-461.

Damiani, C. E. N., Moreira, C. M., Zhang, H. T., Creazzo, T. L. & Vassallo, D. V. (2004). Effects of eugenol, an essential oil, on the mechanical and electrical activities of cardiac muscle. Journal of Cardiovascular Pharmcology, **44**, 688-695.

Damiani, C. E. N., Rossoni, L. V. & Vassallo, D. V. (2003). Vasorelaxant effects of eugenol on rat thoracic aorta. Vascular Pharmacology, **40** (1), 59-66.

Dänicke, S., Valenta, H. & Döll, S. (2004). On the toxicokinetics and the metabolism of deoxynivalenol (DON) in the pig. Archives of Animal Nutrition, **58** (2), 169-180.

Decuypere, J. A., Dierick, N. A., Vervaeke, I. J. & Henderickx, H. K. (1991). Influence of virginiamycine on the digestive physiology in precaecal re-entrant cannulated pigs. Archives of Animal Nutrition **41**, 373-393.

Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D. & Mauriello, G. (2007). Membrane toxicity of antimicrobial compounds from essential oils. Journal of Agricultural and Food Chemistry, **55** (12), 4863-4870.

Di Pasqua, R., Hoskins, N., Betts, G. & Mauriello, G. (2006). Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. Journal of Agricultural and Food Chemistry, **54** (7), 2745-2749.

Dibner, J. J. & Buttin, P. (2002). Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. Journal of Applied Poultry Research, **11**, 453-463.

Dibner, J. J. & Richards, J. D. (2005). Antibiotic growth-promoters in agriculture: history and mode of action. Poultry Science, **84** (4), 634-643.

Didry, N., Dubreuil, L. & Pinkas, M. (1993). Activité antibactérienne du thymol, du carvacrol et de l'aldéhyde cinnamic seuls ou associés. Pharmazie, **43**, 301-304.

Dierick, N. A., Decuypere, J. A. & Degeyter, I. (2003). The combined use of whole *Cuphea* seeds containing medium chain fatty acids and an exogennous lipase in piglet nutrition. Archives of Animal Nutrition, **57**, 49-63.

Dierick, N. A., Decuypere, J. A., Molly, K., Van Beek, E. & Vanderbeke, E. (2002a). The combined use of triacylglycerols containing medium-chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition I. *In vitro* screening of the release of MCFAs from selected fat sources by s elected exogenous lipolytic enzymes under simulated pig gastric conditions and their effects on the gut flora of piglets. Livestock Production Science, **75**, 129-142.

Dierick, N. A., Decuypere, J. A., Molly, K., Van Beek, E. & Vanderbeke, E. (2002b). The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative to nutritional antibiotics in piglet nutrition II. *In vivo* release of MCFAs in gastric cannulated and slaughtered piglets by endogenous and exogenous lipases; effects on luminal gut flora and growth performance. Livestock Production Science, **76**, 1-26.

Dierick, N. A., Decuypere, J. A., Vervaeke, I. J. & Henderickx, H. K. (1981). Resorption of amino acids from an isolated loop of the pig's small intestine *in vivo*: influence of a nutritional dose of virginiamycine. In: Sasaki *et al.* (eds.), Recent Advances in Germfree Research, p369-372.

Dierick, N. A., Vervaeke, I. J., Decuypere, J. A. & Henderickx, H. K. (1986a). Influence of the gut flora and of some growth-promoting feed additives on nitrogen metabolism in pigs. I. Studies *in vitro*. Livestock Production Science, **14**, 161-176.

Dierick, N. A., Vervaeke, I. J., Decuypere, J. A. & Henderickx, H. K. (1986b). Influence of the gut flora and of some growth-promoting feed additives on nitrogen metabolism in pigs. II. Studies *in vivo*. Livestock Production Science, **14**, 177-193.

Domadia, P., Swarup, S., Bhunia, A., Sivaraman, J. & Dasgupta, D. (2007). Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde. Biochemical Pharmacology, **74** (6), 831-840.

Dorman, H. J. D., Figueiredo, A. C., Barroso, J. G. & Deans, G. (2000). *In vitro* evaluation of antioxidant activity of essential oils and their components. Flavour and Fragrance Journal, **15**, 12-16.

Doyle, M. E. (2001). Alternatives to antibiotic use for growth promotion in animal husbandry. FRI Briefings April 2001; Food Research Institute, University of Wisconsin-Madison, Madison, WI 53706.

Du, W. X., Olsen, C. W., Avena-Bustillos, R. T., McHugh, T. H., Levin, C. E. & Friedman, M. (2008). Storage stability and antibacterial activity against *Escherichia coli* O157:H7 of carvacrol in edible apple films made by two different casting methods. Journal of Agricultural and Food Chemistry, **56** (9), 3082-3088.

Dusan, F., Marian, S., Katarina, D. & Dobroslava, B. (2006). Essential oils - their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. Toxicology in Vitro, **20** (8), 1435-1445.

Elahi, E. N., Wright, Z., Hinselwood, D., Hotchkiss, S. A., Basketter, D. A. & Pease, C. K. (2004). Protein binding and metabolism influence the relative skin sensitization potential of cinnamic compounds. Chemical Research in Toxicology **17** (3), 301-310.

Elgendy, E. M. & Khayyat, S. A. (2008). Oxidation reactions of some natural volatile aromatic compounds: anethole and eugenol. Russian Journal of Organic Chemistry, **44** (6), 823-829.

Erickson, D. L., Nsereko, V. L., Morgavi, D. P., Selinger, L. B., Rode, L. M. & Beauchemin, K. A. (2002). Evidence of quorum sensing in the rumen ecosystem: detection of N-acyl homoserine lactone autoinducers in ruminal contents. Canadian Journal of Microbiology, **48** (4), 374-378.

Ettayebi, K., Yamani, J. E. & Rossi-Hassani, B.-D. (2000). Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. FEMS Microbiology Letters, **183** (1), 191-195.

European Commission (2002). Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (http://europa.eu.int/eur-lex/en/archive/2002/l_22120020817en.html). Official Journal of the European Community, **L221**, 8-36.

Evans, W. C. (2002). Trease and Evans. Pharmacognosy. 15th Edition, W.B. Saunders, Edinburgh, UK, 585p.

Eyssen, H. (1973). Role of gut microflora in metabolism of lipids and sterols. Proceedings of the Nutrition Society, **32**, 59-63.

Falcone, P. M., Mastromatteo, M., Del Nobile, M. A., Corbo, M. R. & Sinigaglia, M. (2007). Evaluating *in vitro* antimicrobial activity of thymol toward hygiene-indicating and pathogenic bacteria. Journal of Food Protection, **70** (2), 425-431.

Friedman, M. (1996). Food browning and its prevention: an overview. Journal of Agricultural and Food Chemistry, **44** (3), 631-653.

Friedman, M., Henika, P. R. & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica.* Journal of Food Protection, **65** (10), 1545-1560.

Friedman, M., Kozukue, N. & Harden, L. A. (2000). Cinnamaldehyde content in foods determined by gas chromatografy - mass spectrometry. Journal of Agricultural and Food Chemistry, **48**, 5702-5709.

Fujisawa, S., Atsumi, T., Kadoma, Y. & Sakagami, H. (2002). Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Toxicology, **177** (1), 39-54.

Gaskins, H. R., Collier, C. T. & Anderson, D. B. (2002). Antibiotics as growth promotants: mode of action. Animal Biotechnology, **13** (1), 29 - 42.

Gaysinsky, S., Davidson, P. M., Bruce, B. D. & Weiss, J. (2005a). Growth inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by carvacrol and eugenol encapsulated in surfactant micelles. Journal of Food Protection, **68** (12), 2559-2566.

Gaysinsky, S., Davidson, P. M., Bruce, B. D. & Weiss, J. (2005b). Stability and antimicrobial efficiency of eugenol encapsulated in surfactant micelles as affected by temperature and pH. Journal of Food Protection, **68** (7), 1359-1366.

Gholivand, M. B. & Ahmadi, F. (2008). Simultaneous determination of *trans*-cinnamaldehyde and benzaldehyde in different real samples by differential pulse polarography and study of heat stability of *trans*-cinnamaldehyde. Analytical Letters, **41** (18), 3324-3341.

Gibson, J. & Harwood, C. S. (2002). Metabolic diversity in aromatic compound utilization by anaerobic microbes. Annual Review of Microbiology, **56**, 345-369.

Gill, A. O. & Holley, R. A. (2004). Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei.* Applied and Environmental Microbiology, **70**, 5750-5755.

Gill, A. O. & Holley, R. A. (2006a). Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. International Journal of Food Microbiology, **108** (1), 1-9.

Gill, A. O. & Holley, R. A. (2006b). Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. International Journal of Food Microbiology, **111** (2), 170-174.

Gill, P., Fowler, V. & Armstrong, D. (2005). Alternatives to antibiotic feed additives for pigs, British Society for Animal Science, accessed online 9-27-2005 at http://www.bsas.org.uk/about_the_bsas/issue_papers.

Gollnisch, K. & Halle, I. (2001). Effects of essential oils and herbs in animal nutrition. In: Proceedings of the 8th Symposium on Vitamines and Additives in Nutrition of Man and Animal, September 26th and 27th, Jena, Germany, p32.

Gong, J., Yu, H., Liu, T., Li, M., Si, W., de Lange, C. F. M. & Dewey, C. (2008). Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. Livestock Science, **116** (1-3), 318-322.

Gössling, A. (2001). Wirkungen eines Oreganoöl-Zusatzes als Futteradditiv auf die Darmflora von Absetzferkeln. Dissertation, Tierärztlichen Hochschule Hannover, Deutschland, 137 p.

Gowder, S. J. T. & Devaraj, H. (2006). Effect of the food flavour cinnamaldehyde on the antioxidant status of rat kidney. Basic & Clinical Pharmacology & Toxicology, **99** (5), 379-382.

Greule, M., Hansel, C., Bauermann, U. & Mosandl, A. (2008). Feed additives: authenticity assessment using multicomponent-/multielement-isotope ratio mass spectrometry. European Food Research and Technology, **227** (3), 767-776.

Griffin, S. G., Wylie, S. G., Markham, J. L. & Leach, D. N. (1999). The role of structure and molecular properties of terpenoids in determing their antimicrobial activity. Flavour and Fragrance Journal, **14**, 322-332.

Guenette, S. A., Beaudry, F., Marier, J. F. & Vachon, P. (2006). Pharmacokinetics and anaesthetic activity of eugenol in male Sprague-Dawley rats. Journal of Veterinary Pharmacology and Therapeutics, **29** (4), 265-270.

Guenette, S. A., Ross, A., Marier, J. F., Beaudry, F. & Vachon, P. (2007). Pharmacokinetics of eugenol and its effects on thermal hypersensitivity in rats. European Journal of Pharmacology, **562** (1-2), 60-67.

Günther, K. D. & Bossow, H. (1998). The effect of etheric oil from *Origanum vulgare* (Ropadiar) in the feed ration of weaned pigs on their daily feed intake, daily gains and food utilization. In: Proceedings of the 15th IPVS Congress, Birmingham, UK, 5-9 July 1998. . 223.

Guo, J.-Y., Huo, H.-R., Zhao, B.-S., Liu, H.-B., Li, L.-F., Ma, Y.-Y., Guo, S.-Y. & T.-L., J. (2006). Cinnamaldehyde reduces IL-1β-induced cyclooxygenase-2 activity in rat cerebral microvascular endothelial cells. European Journal of Pharmacology, **537**, 174-180.

Gutierrez, J., Barry-Ryan, C. & Bourke, R. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. International Journal of Food Microbiology, **124** (1), 91-97.

Guy-Grand, D., Di Santo, J. P., Henchoz, P., Malassis-Seris, M. & Vassalli, P. (1998). Small bowel enteropathy: role of intraepithelial lymphocytes and of cytokines (IL-12, IFN-γ, TNF) in the induction of epithelial cell death and renewal. European Journal of Immunology, **28**, 730-744.

Haarmann & Reimer (2001). Ready biodegradability of cinnamic aldehyde according to OECD guideline No. 301B. Private Communication to RIFM.

Hagmuller, W., Jugl-Chizzola, M., Zitterl-Eglseer, K., Gabler, C., Spergser, J., Chizzola, R. & Franz, C. (2006). The use of Thymi Herba as feed additive (0.1%, 0.5%, 1.0%) in weanling piglets with assessment of the shedding of haemolysing *E. coli* and the detection of thymol in the blood plasma. Berliner Und Münchener Tierarztliche Wochenschrift, **119** (1-2), 50-54.

Harada, M. & Yano, S. (1975). Pharmalogical studies on Chinese cinnamon. II. Effects of cinnamaldehyde on the cardiovascular and digestive systems. Chemical & Pharmaceutical Bulletin, **23** (5), 941-947.

Haselmeyer, A. (2007). Wirkung von Thymian als Futterzusatz beim Broiler. Inaugural-Dissertation zur Erlangung des Würde eines Doctor Medicinale Veterinariae der Veterinärmedizinischen Universität Wein, 157p.

Hathaway, M. R., Dayton, W. R., White, M. E., Henderson, T. L. & Henningson, T. B. (1996). Serum insulin-like growth factor I (IGF-I) concentrations are increased in pigs fed antimicrobials. Journal of Animal Science, **74** (7), 1541-1547.

Hausner, H., Bredie, W. L. P., Mølgaard, C., Petersen, M. A. & Møller, P. (2008). Differential transfer of dietary flavour compounds into human breast milk. Physiology & Behavior, **95** (1-2), 118-124.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., Gorris, L. G. M. & Von Wright, A. (1998). Characterization of the action of selected essential oil components on gram-negative bacteria. Journal of Agricultural and Food Chemistry, **46**, 3590-3595.

Hojberg, O., Canibe, N., Poulsen, H. D., Hedemann, M. S. & Jensen, B. B. (2005). Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Applied Environmental Microbiology, **71** (5), 2267-2277.

Hu, D. F. & Coats, J. (2008). Evaluation of the environmental fate of where science meets business thymol and phenethyl propionate in the laboratory. Pest Management Science, **64** (7), 775-779.

Huang, J., Wang, S., Luo, X., Xie, Y. & Shi, X. (2007). Cinnamaldehyde reduction of platelet aggregation and thrombosis in rodents. Thrombosis Research, **119** (3), 337-342.

Hulin, V., Mathot, A.-G., Mafart, P. & Dufossé, L. (1998). Les propriétés anti-microbiennes des huiles essentielles et composés d' arômes. Sciences des Aliments, **18**, 563-582.

Huss, U., Ringbom, T., Perera, P., Bohlin, L. & Vasange, M. (2002). Screening of ubiquitous plant constituents for COX-2 inhibition with a scintillation proximity based assay. Journal of Natural Products, **65** (11), 1517-1521.

Ilsley, S. E., Miller, H. M., Greathead, H. M. R. & Kamel, C. (2003). Plant extracts as supplements for lactating sows: effects on piglet performance, sow food intake and diet digestibility. Animal Science, **77**, 247-254.

Imdorf, A., Bogdanov, S., Ochoa, R. I. & Calderone, N. W. (1999). Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies. Apidologie, **30** (2-3), 209-228.

Imparl-Radosevich, J., Deas, S., Polansky, M. M., Baedke, D. A., Ingebritsen, T. S., Anderson, R. A. & Graves, D. J. (1998). Regulation of PTP-1 and insulin receptor kinase by fractions from cinnamon: implications for cinnamon regulation of insulin signalling. Hormone Research, **50** (3), 177-182.

Inouye, S., Takizawa, T. & Yamaguchi, H. (2001). Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. Journal of Antimicrobial Chemotherapy, **47** (5), 565-573.

Ipek, E. J., Zeytinoglu, H., Okay, S., Tuylu, B. A., Kurkcuoglu, M. & Can Baser, K. H. (2005). Genotoxicity and antigenotoxicity of origanum oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. Food Chemistry, **93**, 551-556.

Ito, M., Murakami, K. & Yoshino, M. (2005). Antioxidant action of eugenol compounds: role of metal ion in the inhibtion of lipid peroxidation. Food Chemical Toxicology, **43**, 461-466.

Janczyk, P., Trevisi, P., Souffrant, W. B. & Bosi, P. (2008). Effect of thymol on microbial diversity in the porcine jejunum. International Journal of Food Microbiology, **126** (1-2), 258-261.

Janz, J. A. M., Morel, P. C. H., Wilkinson, B. H. P. & Purchas, R. W. (2007). Preliminary investigation of the effects of low-level dietary inclusion of fragrant essential oils and oleoresins on pig performance and pork quality. Meat Science, **75** (2), 350-355.

Janzowski, C., Glaab, V., Mueller, C., Straesser, U., Kamp, H. G. & Eisenbrand, G. (2003). Alfa,beta-unsaturated carbonyl compounds: induction of DNA damage in mammalian cells. Mutagenesis, **18** (5), 465-470.

Jensen, B. B. & Mikkelsen, L. L. (1998). Feeding liquid diets to pigs. In: Garnsworthy, P.C. & Wiseman, J. (Eds), Recent Advances in Animal Nutrition, The Nottingham University Press, Nottingham, UK. p107-126.

Jensen, B.B. (1998). The impact of feed additives on the microbial ecology of the gut in young pigs. Journal of Animal and Feed Science, **7**, 45-64.

Jensen, M. T., Cox, R. P. & Jensen, B.B. (1995). Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. Animal Science **61**, 293-304.

Jones, F. A. (1996). Herbs - useful plants. Their role in history and today. European Journal of Gastroenterology & Hepatology, **8** (12), 1227-1231.

Jongbloed, A. W., Maiorano, R. & Wagenaars, C. M. F. (2007). Effect of several plant products on prevention of E. coli adhesion in the gastrointestinal tract of weaned piglets. Rapport 62, Divisie Veehouderij, Animal Sciences Group, Wageningen, UR, 29p.

Jugl-Chizzola, M., Spergser, J., Schilcher, F., Novak, J., Bucher, A., Gabler, C., Hagmuller, W. & Zitterl-Eglseer, K. (2005). Effects of *Thymus vulgaris* L. as feed additive in piglets and against haemolytic *E. coli in vitro*. Berliner und Münchener Tierarztliche Wochenschrift, **118** (11-12), 495-501.

Jugl-Chizzola, M., Ungerhofer, E., Gabler, C., Hagmuller, W., Chizzola, R., Zitterl-Eglseer, K. & Franz, C. (2006). Testing of the palatability of *Thymus vulgaris* L. and *Origanum vulgare* L. as flavouring feed additive for weaner pigs on the basis of a choice experiment. Berliner und Münchener Tierarztliche Wochenschrift, **119** (5-6), 238-243.

Jukic, M., Politeo, O., Maksimovic, M., Milos, M. & Milos, M. (2007). *In vitro* acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phytotherapy Research, **21** (3), 259-261.

Juven, B. J., Kanner, J., Schved, F. & Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. Journal of Applied Bacteriology, **76**, 626-631.

Ka, H., Park, H.-J., Jung, H.-J., Choi, J.-W., Cho, K.-S., Ha, J. & Lee, K.-T. (2003). Cinnamaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. Cancer Letters, **196**, 143-152.

Kabuto, H., Tada, M. & Kohno, M. (2007). Eugenol [2-methoxy-4-(2-propenyl)phenol] prevents 6-hydroxydopamine-induced dopamine depression and lipid peroxidation inductivity in mouse striatum. Biological & Pharmaceutical Bulletin, **30** (3), 423-427.

Kamel, C., 2001. Tracing modes of action and the roles of plant extracts in non-ruminants. In: Garnsworthy, P.C., Wiseman, J. (Eds), Recent Advances in Animal Nutrition. The Nottingham University Press, Nottingham, p135–150.

Karapinar, M. & Aktug, S. E. (1987). Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole. International Journal of Food Microbiology, **4**, 161-166.

Karpanen, T. J., Worthington, T., Hendry, E. R., Conway, B. R. & Lambert, P. A. (2008). Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*. Journal of Antimicrobial Chemotherapy, **62** (5), 1031-1036.

Kellett, G. L. & Helliwell, P. A. (2000). The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. Biochemical Journal, **350**, 155-162.

Kim, D. H., Kim, C. H., Kim, M. S., Kim, J. Y., Jung, K. J., Chung, J. H., An, W. G., Lee, J. W., Yu, B. P. & Chung, H. Y. (2007). Suppression of age-related inflammatory NF-kappa B activation by cinnamaldehyde. Biogerontology, **8** (5), 545-554.

Kim, H.-O., Park, S.-W. & Park, H.-D. (2004). Inactivation of *Escherichia coli* O157:H7 by cinnamic aldehyde purified from Cinnamomum cassia shoot. Food Microbiology, **21**, 105-110.

Kim, J., Marshall, M. R. & Wei, C.-i. (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry, **43**, 2839-2845.

Kim, K. M., Hanna, M. A., Weller, C. L., Cho, S. H. & Choi, S. G. (2006). Characterization of cinnamaldehyde-supplemented soy protein isolate films. Food Science and Biotechnology, **15** (4), 631-634.

Kis, R. K. & Bilkei, G. (2003). Effect of a phytogenic feed additive on weaning-to-estrus interval and farrowing rate in sows. Journal of Swine Health and Production, **11** (5), 296-299.

Klasing, K. C. (2004). The costs of immunity. Acta Zoologica Sinica, **50** (6), 961-969.

Klasing, K. C. (2007). Nutrition and the immune system. British Poultry Science, **48** (5), 525 - 537.

Klasing, K. C., Laurin, D. E., Peng, R. K. & Fry, D. M. (1987). Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. Journal of Nutrition, **117** (9), 1629-1637.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H. & Weis, N. (1989). Antibacterial and antifungal properties of essential oil components. Journal of Essential Oil Research, **1**, 119-128.

Kobayashi, Y., Suzuki, M., Satsu, H., Arai, S., Hara, Y., Suzuki, K., Miyamoto, Y. & Shimizu, M. (2000). Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. Journal of Agricultural and Food Chemistry, **48** (11), 5618-5623.

Koh, W. S., Yoon, S. Y., Kwon, B. M., Jeong, T. C., Nam, K. S. & Han, M. Y. (1998). Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. International Journal on Immunopharmacology, **20**, 643-660.

Kohlert, C. (2001). Systemische Verfügbarkeit und Pharmakokinetik von Thymol nach oraler Applikation einer thymianhaltigen Zubereitung im Menschen. Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Bayerischen Julius-Maximilians-Üniversität Würzburg, 166p.

Kohlert, C., Schindler, G., März, R. W., Abel, G., Brinkhaus, B., Derendorf, H., Gräfe, E.-U. & Veit, M. (2002). Systemic availibilty and pharmacokinetics of thymol in humans. Journal of Clinical Pharmacology, **42**, 731-737.

Kohlert, C., Van Rensen, I., März, R., Schindler, G., Graefe, E. U. & Veit, M. (2000). Bioavailability and pharmacokinetics of natural volatile terpenes in animals and humans. Planta Medica, **6**, 495-505.

Kreydiyyeh, S. I., Usta, J. & Copti, R. (2000). Effect of cinnamon, clove and some of their constituents on the Na+-K+-ATPase activity and alanine absorption in the rat jejunum. Food and Chemical Toxicology, **38**, 755-762.

Kreydiyyeh, S. I., Usta, J., Knio, K., Markossian, S. & Dagher, S. (2003). Aniseed oil increases glucose absorption and reduces urine output in the rat. Life Sciences, **74** (5), 663-673.

Krist, S., Halwachs, L., Sallaberger, G. & Buchbauer, G. (2007). Effects of scents on airborne microbes, part I: thymol, eugenol, *trans*-cinnamaldehyde and linalool. Flavour and Fragrance Journal, **22** (1), 44-48.

Kubo, I., Fujita, K. & Nihei, K. (2008). Antimicrobial activity of anethole and related compounds from aniseed. Journal of the Science of Food and Agriculture, **88** (2), 242-247.

Kulisic, T., Radonic, A., Katalinic, V. & Milos, M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil. Food Chemistry, **85** (4), 633-640.

Kwon, J. A., Yu, C. B. & Park, H. D. (2003). Bacteriocidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. Letters in Applied Microbiology, **37**, 61-65.

Kyriakis, S. C., Sarris, K., Lekkas, S., Tsinas, A. C., Ginnakopoulos, C. G., Alexopoulos, C. & Saoulidis, K. (1998). Control of post weaning diarrhoea syndrome of piglets by in-feed applications or Origanum essential oils. In: Proceedings of the 15th IPVS Congress, Birmingham, UK, 5-9 July 1998, **3**, 218.

Laitat, M., De Jaeger, F., Vandenheede, M. & Nicks, B. (2004). Facteurs influençant la consommation alimentaire et les performances zootechniques du porc sevré: perception et caractéristiques de l'aliment Annales des Médicine Vétérinaire, **148**, 15-29.

Lallès, J. P., Boudry, G., Favier, C., Le Floc'H, N., Luron, I. M., Montagne, L., Oswald, I. P., Pié, S., Piel, C. & Sève, B. (2004). Gut function and dysfunction in young pigs: physiology. Animal Research, **53**, 301-316.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J. & Nychas, G.-J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology, **91**, 453-462.

Landa, P., Kokoska, L., Pribylova, M., Vanek, T. & Marsik, P. (2009). *In vitro* anti-inflammatory activity of carvacrol: inhibitory effect on COX-2 catalyzed prostaglandin E-2 biosynthesis. Archives of Pharmacological Research, **32** (1), 75-78.

Leach, D. N., Wyllie, S. G., Hall, J. G. & Kyratzis, I. (1993). Enatiomeric composition of the principal components of the oil of *Melaleuca alternifolia.* Journal of Agricultural Food Chemistry, **41**, 1627-1632.

Leal-Cardoso, J. H., Lahlou, S., Coelho-de-Souza, A. N., Criddle, D. N., Pinto, G. I. B., Santos, M. A. V. & Magalhaes, P. J. C. (2002). Inhibitory actions of eugenol on rat isolated ileum. Canadian Journal of Physiological Pharmacology, **80**, 901-906.

Lee, C. W., Lee, S. H., Lee, J. W., Ban, J. O., Lee, S. Y., Yoo, H. S., Jung, J. K., Moon, D. C., Oh, K. W. & Hong, J. T. (2007). 2-hydroxycinnamaldehyde inhibits SW620 colon cancer cell growth through AP-1 inactivation. Journal of Pharmacological Sciences, **104** (1), 19-28.

Lee, H.-S. & Ahn, Y.-J. (1998). Growth-inhibiting effects of Cinnamomum cassia bark-derived materials on human intestinal bacteria. Journal of Agricultural and Food Chemistry, **46**, 8-12.

Lee, H.-S. (2002). Inhibitory activity of Cinnamomum cassia bark-derived component against rat lens aldose reductase. Journal of Pharmacy and Pharmaceutical Sciences, **5** (3), 226-230.

Lee, H.-S., Beon, M.-S. & Kim, M.-K. (2001). Selective growth inhibitor toward human intestinal bacteria derived from *Pulsatilla cernua* root. Journal of Agricultural and Food Chemistry, **49**, 4656-4661.

Lee, K.-W., Everts, H. & Beynen, A. C. (2004). Essential oils in broiler nutrition. International Journal of Poultry Science, **3** (12), 738-752.

Lee, S. P., Buber, M. T., Ceme, R., Cortes, R. Y. & Bryant, R. W. (2007a). Thymol and related phenols are potent activators of the transient receptor potential channel, TRPA1. Faseb Journal, **21** (6), A1158-A1158.

Lee, S.-Y. & Jin, H. H. (2008). Inhibitory activity of natural antimicrobial compounds alone or in combination with nisin against *Enterobacter sakazakii*. Letters in Applied Microbiology, **47** (4), 315-321.

Lee, Y. Y., Hung, S. L., Pai, S. F., Lee, Y. H. & Yang, S. F. (2007b). Eugenol suppressed the expression of lipopolysaccharide-induced proinflammatory mediators in human macrophages. Journal of Endodontics, **33** (6), 698-702.

Lima, C. C., Criddle, D. N., Coelho-de-Souza, A. N., Monte, F. J. Q., Jaffar, M. & Leal-Cardoso, J. H. (2000). Relaxant and antispasmodic actions of methyleugenol on guinea-pig isolated ileum. Planta Medica, **66** (5), 408-411.

Lis-Balchin, M. (2003). Feed additives as alternatives to antibiotic growth-promoters: botanicals. In: Proceedings of the 9th International Symposium on Digestive Physiology in Pigs, Banff, AB, Canada. Volume 1, p333-352.

Lodemann, U., Hubener, K., Jansen, N. & Martens, H. (2006). Effects of *Enterococcus faecium* NCIMB 10415 as probiotic supplement on intestinal transport and barrier function of piglets. Archives of Animal Nutrition, **60** (1), 35-48.

Lopez, P., Sanchez, C., Batlle, R. & Nerin, C. (2007). Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. Journal of Agricultural and Food Chemistry, **55** (11), 4348-4356.

Macheboeuf, D., Morgavi, D. P., Papon, Y., Mousset, J. L. & Arturo-Schaan, M. (2008). Dose-response effects of essential oils on *in vitro* fermentation activity of the rumen microbial population. Animal Feed Science and Technology, **145** (1-4), 335-350.

Macpherson, L. J., Hwang, S. W., Miyamoto, T., Dubin, A. E., Patapoutian, A. & Story, G. M. (2006). More than cool: promiscuous relationships of menthol and other sensory compounds. Molecular and Cellular Neuroscience, **32** (4), 335-343.

Macpherson, L. J., Xiao, B., Kwan, K. Y., Petrus, M. J., Dubin, A. E., Hwang, S., Cravatt, B., Corey, D. P. & Patapoutian, A. (2007). An ion channel essential for sensing chemical damage. Journal of Neuroscience, **27** (42), 11412-11415.

Magyar, J., Szentandrassy, N., Banyasz, T., Fulop, L., Varro, A. & Nanasi, P. P. (2002). Effects of thymol on calcium and potassium currents in canine and human ventricular cardiomyocytes. British Journal of Pharmacology, **136** (2), 330-338.

Magyar, J., Szentandrassy, N., Banyasz, T., Fulop, L., Varro, A. & Nanasi, P. P. (2004). Effects of terpenoid phenol derivatives on calcium current in canine and human ventricular cardiomyocytes. European Journal of Pharmacology, **487** (1-3), 29-36.

Manzanilla, E. G. (2005). Evaluation of in-feed additives in early-weaned pigs: study of the XTRACT, a plant extracts based additive. Per accedir al Grau de Doctor dins el programma de doctorat de produccio animal de Departement de Ciencia Animal i dels Aliments. Bellaterra, 2005, Spain.

Manzanilla, E. G., Perez, J. F., Martin, M., Kamel, C., Baucells, F. & Gasa, J. (2004). Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. Journal of Animal Science, **82**, 3210-3218.

Marsik, P., Kokoska, L., Landa, P., Nepovim, A., Soudek, P. & Vanek, T. (2005). *In vitro* inhibitory effects of thymol and quinones of *Nigella sativa* seeds on cyclooxygenase-1-and-2-catalyzed prostaglandin E-2 biosyntheses. Planta Medica, **71** (8), 739-742.

Marsik, P., Landa, P., Pribylova, M., Vanek, T. & Kokoska, L. (2006). Carvacrol as the inhibitor of cyclooxygenase-1 and -2, the key enzymes of prostaglandin biosynthesis: *in vitro* assays. In: Proceedings of the 54th Annual Congress on Medicinal Plant Research, 29 August - 2 September 2006, Helsinki, Finland. Planta Medica, **72** (11), 1108.

Mastelic, J. & Jerkovic, I. (2003). Gas chromatography-mass spectrometry analysis of free and glycoconjugated aroma compounds of seasonally collected *Satureja montana* L. Food Chemistry, **80** (1), 135-140.

Mastelic, J., Jerkovic, I., Blazevic, I., Poljak-Blazi, M., Borovic, S., Ivancic-Bace, I., Smrecki, V., Zarkovic, N., Brcic-Kostic, K., Vikic-Topic, D. & Muller, N. (2008). Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. Journal of Agricultural and Food Chemistry, **56** (11), 3989-3996.

Mathew, A. G., Upchurch, W. G. & Chattin, S. E. (1998). Incidence of antibiotic resistance in fecal *Escherichia coli* isolated from commercial swine farms. Journal of Animal Science, **76** (2), 429-434.

Mauch, C. & Bilkei, G. (2004). Strategic application of oregano feed supplements reduces sow mortality and improves reproductive performance - a case study. Journal of Vetarinary Pharmacological Therapy, **27**, 61-63.

McCarthy, J.F., Aherne, F.X. & Okai, D.B. (1974). Use of HCl insoluble ash as an index material for determining apparent digestibility with pigs. Canadian Journal of Animal Science, **54**, 107-109.

McLaughlin, C. L., Baile, C. A., Buckholtz, L. L. & Freeman, S. K. (1983). Preferred flavours and performance of weanling pigs. Journal Animal Science, **56** (6), 1287-1293.

Meunier, J.-P., Cardot, J.-M., Gauthier, P., Beyssac, E. & Alric, M. (2006). Use of rotary fluidized-bed technology for development of sustained-release plants extracts pellets: potential application for feed additive delivery. Journal of Animal Science, **84**, 1850-1859.

Michiels, J., Missotten, J. A. M., Van Hoorick, A., Ovyn, A., Fremaut, D., De Smet, S. & Dierick, N. A. (revised version submitted). Effect of dose and formulation of carvacrol and thymol on gut bacteria and gut function in piglets after weaning. Animal.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (submitted). Thymol and *trans*-cinnamaldehyde reduce active nutrient absorption and stimulated chloride secretion in the pig jejunal Ussing chamber model. Livestock Science.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D., Maene, P. & De Smet, S. (2008). *In vitro* degradation and *in vivo* passage kinetics of carvacrol, thymol, eugenol and *trans*-cinnnamaldehyde along the gastrointestinal tract of piglets. Journal of the Science of Food and Agriculture **88**, 2371-2381.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. A. (2007). *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. Livestock Science, **109**, 157-160.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. A. (2009). *In vitro* characterisation of the antimicrobial activity of selected essential oil components and binary combinations against the pig gut flora. Animal Feed Science and Technology, doi:10.1016/j.anifeedsci.2009.01.004

Mikkelsen, L. L. & Jensen, B.B. (2004). Effect of fructo-oligosaccharides and transgalacto-oligosaccharides on microbial populations and microbial activity in the gastrointestinal tract of piglets post-weaning. Animal Feed Science and Technology, **117**, 107-119.

Milos, M., Mastelic, J. & Jerkovic, I. (2000). Chemical composition and antioxidant effect of glycosidically bound volatile compounds from oregano (*Origanum vulgare* L. ssp hirtum). Food Chemistry, **71** (1), 79-83.

Möller, T. (2001). Untersuchungen zum Einfluss eines Oreganoöl-Zusatzes zum Futter auf die Rohnärstoffverdaulichkeit, N-Bilanz sowie auf Parameter des mikrobiellen Stoffwechsels im Verdauungstrakt von Absetzferkeln. Dissertation, Tierärztlichen Hochschule Hannover, Deutschland.

Molnar, C. & Bilkei, G. (2005). The influence of an oregano feed additive on production parameters and mortality of weaned piglets. Tierärztliche Praxis, Ausgabe Großtiere, Nutztiere, **33**, 42–47.

Montagne, L., Boudry, G., Favier, C., Le Huerou-Luron, I., Lalles, J. P. & Seve, B. (2007). Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. British Journal of Nutrition, **97** (1), 45-57.

Morsy, M. A. & Fouad, A. A. (2008). Mechanisms of gastroprotective effect of eugenol in indomethacin-induced ulcer in rats. Phytotherapy Research, **22** (10), 1361-1366.

Mühlbuer, R. C., Lozano, A., Palacio, S., Reinli, A. & Felix, R. (2003). Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. Bone, **32**, 372-380.

Murakami, Y., Hirata, A., Ito, S., Shoji, M., Tanaka, S., Yasui, T., Machino, M. & Fujisawa, S. (2007). Re-evaluation of cyclooxygenase-2-inhibiting activity of vanillin and guaiacol in macrophages stimulated with lipopolysaccharide. Anticancer Research, **27** (2), 801-807.

Nakada, N., Shinohara, H., Murata, A., Kiri, K., Managaki, S., Sato, N. & Takada, H. (2007). Removal of selected pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs) during sand filtration and ozonation at a municipal sewage treatment plant. Water Research, **41** (19), 4373-4382.

Namer, B., Seifert, F., Handwerker, H. O. & Maihofner, C. (2005). TRPA1 and TRPM8 activation in humans: effects of cinnamaldehyde and menthol. Neuroreport, **16** (9), 955-959.

Namkung, H., Li, M., Gong, J., Yu, H., Cottrill, M. & De Lange, C. F. M. (2004). Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. Canadian Journal of Animal Science, **84**, 697-704.

Nazer, A. I., Kobilinsky, A., Tholozan, J.-L. & Dubois-Brissonnet, F. (2005). Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* serovar Typhimurium: a synergistic effect? Food Microbiology, **22**, 391-398.

Neill, C. R., Nelssen, J. L., Tokach, M. D., Goodband, R. D., Dritz, S. S. & DeRouchey, M. (2006). Effects of increasing oregano oils in nursery pig diets. Journal of Animal Science, **84,** Suppl. 2, 63.

Nhu-Trang, T. T., Casabianca, H. & Grenier-Loustalot, M. F. (2006). Deuterium/hydrogen ratio analysis of thymol, carvacrol, gamma-terpinene and *p*-cymene in thyme, savory and oregano essential oils by gas chromatography-pyrolysis-isotope ratio mass spectrometry. Journal of Chromatografy A, **1132** (1-2), 219-227.

Niewold, T. A. (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A Hypothesis. Poultry Science, **86** (4), 605-609.

Nishijima, H., Uchida, R., Kameyama, K., Kawakami, N., Ohkubo, T. & Kitamura, K. (1999). Mechanisms mediating the vasorelaxing action of eugenol, a pungent oil, on rabbit arterial tissue. Japanese Journal of Pharmacology, **79** (3), 327-334.

Niu, C., Afre, S. & Gilbert, E. S. (2006). Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. Letters in Applied Microbiology, **43** (5), 489-494.

Nofrarias, M., Manzanilla, E. G., Pujols, J., Gibert, X., Majo, N., Segales, J. & Gasa, J. (2006). Effects of spray-dried porcine plasma and plant extracts on intestinal morphology and on leukocyte cell subsets of weaned pigs. Journal of Animal Science, **84** (10), 2735-2742.

Nostro, A., Blanco, A. R., Cannatelli, M. A., Enea, V., Flamini, G., Morelli, I., Sudano Roccaro, A. & Alonzo, V. (2004). Susceptibility of methicillin-resistant *Staphylococci* to oregano essential oil, carvacrol and thymol. FEMS Microbiology Letters, **230** (2), 191-195.

Nostro, A., Roccaro, A. S., Bisignano, G., Marino, A., Cannatelli, M. A., Pizzimenti, F. C., Cioni, P. L., Procopio, F. & Blanco, A. R. (2007). Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Journal of Medical Microbiology, **56** (4), 519-523.

Nozawa, K., Kawabata-Shoda, E., Doihara, H., Kojima, R., Okada, H., Mochizuki, S., Sano, Y., Inamura, K., Matsushime, H., Koizumi, T., Yokoyama, T. & Ito, H. (2009). TRPA1 regulates gastrointestinal motility through serotonin release from enterochromaffin cells. Proceedings of the National Academy of Sciences, doi:10.1073/pnas.0805323106.

Nychas, G.-J. E. & Skandamis, P. N. (2003). Antimicrobials from herbs and spices. In: Roller, S. (Ed.), Natural antimicrobials for the minimal processing of foods. Woodhead Publishing Limited, Cambridge. p176-200.

Oetting, L. L., Utiyama, C. E., Giani, P. A., Ruiz, U. D. & Miyada, V. S. (2006a). Effects of antimicrobials and herbal extracts on intestinal microbiology and diarrhea incidence in weanling pigs. Brazilian Journal of Animal Science, **35** (5), 2013-2017.

Oetting, L. L., Utiyama, C. E., Giani, P. A., Ruiz, U. D. & Miyada, V. S. (2006b). Effects of herbal extracts and antimicrobials on apparent digestibility, performance, organs morphometry and intestinal histology of weanling pigs. Brazilian Journal of Animal Science, **35** (4), 1389-1397.

Ogata, M. (2008). Antioxidant and antibacterial activities of dimeric phenol compounds. Journal of the Pharmaceutical Society of Japan, **128** (8), 1149-1158.

Ogata, N., Matsushima, N. & Shibata, T. (1995). Pharmacokinetics of wood creosote - glucuronic-acid and sulfate conjugation of phenolic-compounds. Pharmacology, **51** (3), 195-204.

Okada, N., Hirata, A., Murakami, Y., Shoji, M., Sakagami, H. & Fujisawa, S. (2005). Induction of cytotoxicity and apoptosis and inhibition of cyclooxygenase-2 gene expression by eugenol-related compounds. Anticancer Research, **25** (5), 3263-3269.

Olasupo, N. A., Fitzgerald, D. J., Gasson, M. J. & Narbad, A. (2003). Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Letters of Applied Microbiology, **37** (6), 448-451.

Orhan, I., Kartal, M., Kan, Y. & Sener, B. (2008). *In vitro* testing of selected essential oils and various pure components against acetylcholinesterase and butyrylcholinesterase. Zeitschrift Für Naturforschung, Section C-a, **63** (7-8), 547-553.

Oswald, J., Zitterl-Eglseer, K. & Wetscherek, W. (2007). Transfer von Thymol und Carvacrol aus dem Futter in die Milch von Sauen sowie die Auswirkung auf die Entwicklung der Saugferkel. In: Proceedings of the 6-BOKU Symposium Tierernährung, 15. November 2005, Wien, Austria, p. 183-186.

Page, S. W. (2006). Current use of antimicrobial growth promoters in food animals: the benifits. In: Barug, D., De Jong, J., Kies, A.K., Verstegen, M.W.A. (Eds), Antimicrobial growth promoters. Where do we go from here? Wageningen Academic Publishers, Wageningen, pp.19-51.

Pauli, A. & Schilcher, H. (2004). Specific selection of essential oils compounds for treatment of children's infection disease. Pharmaceuticals, **1**, 1-30.

Pedroso, A. A., Oetting, L. L., Utiyama, C. E., Menten, J. F. M., Lambais, M. R. & Miyada, V. S. (2005). Spacial variability of intestinal bacterial population of swine supplemented with antimicrobial or herbal extracts. Brazilian Journal of Animal Science, **34** (4), 1225-1233.

Peňalver, P., Huerta, B., Borge, C., Astorga, R., Romero, R. & Perea, A. (2005). Antimicrobial activity of five essential oils against origin strains of the Enterobacteriaceae family. APMIS, **113**, 1-6.

Pérez, O., De Filippi, S., Akkermans, W., Kluess, J., Bikker, P., Fledderus, J., Pellikaan, W., Verstegen, M., Smidt, H. & De Vos, W. M. (2007). Effect of oregano oil constituent on the gut microbiota of weaning piglets. In Proceedings of the 9th Annual GutDay, 14th November 2007, Wageningen, The Netherlands.

Periago, P. M., Palop, A. & Fernandez, P. S. (2001). Combined effect of nisin, carvacrol and thymol on the viability of *Bacillus cereus* heat-treated vegetative cells. Food Science Technology International, **7** (6), 487-492.

Peters, M. M. C. G. & Caldwell, J. (1994). Studies on *trans*-cinnamaldehyde. 1. The influence of dose size and sex on its deposition in the rat and mouse. Food and Chemical Toxicology, **32** (10), 869-876.

Piva, A., Anfossi, P., Meola, E., Pietri, A., Panciroli, A., Bertuzzi, T. & Formigoni, A. (1997). Effect of microcapsulation on absorption processes in the pig. Livestock Production Science, **51**, 53-61.

Piva, A., Cervellati, C., Call, J. E. & Luchansky, J. B. (2002). Effect of carvacrol on indigenous Enterobacteriaceae levels and fermentation products in an *in vitro* cecal fermentation system. Journal of Animal Science, **80** (Suppl. 1), nr .1585.

Piva, A., Grilli, E., Messina, M. R., Albonetti, S., Pizzamiglio, V., Cipollini, I., Gatta, P. P. & Zaghini, G. (2007b). Citric acid and thymol influence gastrointestinal microflora in pigs at weaning. Poultry Science, **86** (Suppl. 1), 309-309.

Piva, A., Pizzamiglio, V., Morlacchini, M., Tedeschi, M. & Piva, G. (2007a). Lipid micro-encapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. Journal of Animal Science, **85**, 486-493.

Pluske, J. R., Hampson, D. J. & Williams, I. H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livestock Production Science, **51**, 215-236.

Pol, I. E. & Smid, E. J. (1999). Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes.* Letters in Applied Microbiology, **29**, 166-170.

Pol, I. E. (2001). Influence of food matrix on inactivation of *Bacillus cereus* by combinations of nisin, pulsed electric field treatment, and carvacrol. Journal of Food Protection, **64** (7), 1012-1018.

Prasad, N. S., Raghavendra, R., Lokesh, B. R. & Naidu, K. A. (2004). Spice phenolics inhibit human PMNL 5-lipoxygenase. Prostaglandins Leukotriens Essential Fatty Acids, **70** (6), 521-528.

Priestley, C. M., Williamson, E. M., Wafford, K. A. & Sattelle, D. B. (2003). Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. British Journal of Pharmacology, **140** (8), 1363-1372.

Prieto, J. M., Iacopini, P., Cioni, P. & Chericoni, S. (2007). *In vitro* activity of the essential oils of *Origanum vulgare, Satureja montana* and their main constituents in peroxynitrite-induced oxidative processes. Food Chemistry, **104** (3), 889-895.

Priyadarsini, K. I., Guha, S. N. & Rao, M. N. A. (1998). Physico-chemical properties and antioxidant activities of methoxy phenols. Free Radical Biology and Medicine, **24** (6), 933-941.

Pumbwe, L., Skilbeck, C. A. & Wexler, H. M. (2007). Induction of multiple antibiotic resistance in *Bacteroides fragilis* by benzene and benzene-derived active compounds of commonly used analgesics, antiseptics and cleaning agents. Journal of Antimicrobial Chemotherapy, **60** (6), 1288-1297.

Radonic, A. & Milos, M. (2003). Chemical composition and *in vitro* evaluation of antioxidant effect of free volatile compounds from *Satureja montana* L. Free Radical Research, **37** (6), 673 - 679.

Ragland, D., Schneider, J., Stevenson, D., Hill, M. A. & Bakker, M. (2007). Oregano oil as an alternative to antimicrobials in nursery diets. Journal of Swine Health and Production, **15** (6), 346-351.

Raveendran, M., Thanislass, J., Maheswari, G. U. & Devaraj, H. (1993). Induction of prooxidant state by the food flavor cinnamaldehyde in rat-liver. Journal of Nutritional Biochemistry, **4** (3), 181-183.

Ravishankar, S., Zhu, L. B., Law, B., Joens, L. & Friedman, M. (2008). Plant-derived compounds inactivate antibiotic-resistant *Campylobacter jejuni* strains. Journal of Food Protection, **71** (6), 1145-1149.

Reddy, A. M., Seo, J. H., Ryu, S. Y., Kim, Y. S., Kim, Y. S., Min, K. R. & Kim, Y. (2004). Cinnamaldehyde and 2-methoxycinnamaldehyde as NF-κ B inhibitors from *Cinnamomum cassia.* Planta Medica, **70**, 823-827.

Reid, C. A. & Hillman, K. (1999). The effects of retrogradation and amylose/amylopectin ratio of starches on carbohydrate fermentation and microbial populations in the porcine colon. Animal Science, **68**, 503-510.

Report (2006). Reductie van Salmonella-uitscheiding bij het varken: aangepaste voederstrategieën primordiaal voor de Vlaamse Varkenshouderij. ILVO & Lanupro, Eindverslag Okt2004 - Dec2006, Contractueel Landbouwkundig Onderzoek project, IWT/030845, 145p.

Rhayour, K., Bouchikhi, T., Tantaoui-Elaraki, A., Sendide, K. & Remmal, A. (2003). The mechanism of bactericidal action of oregano and clove essential oils and of their phenolic major components on *Escherichia coli* and *Bacillus subtilis.* Journal of Essential Oil Research, **15** (5), 356-362.

Robbers, J. E., Speedie, M. K. & Tyler, V. E. (1996). Pharmacognosy and pharmacobiotechnology, Williams and Wilkins, Baltimore, USA, 337p.

Rodehutscord, M. & Kluth, H. (2002). Tierfütterung ohne antibiotisch wirkende Leistungförderer. Züchtungskunde, **74** (6), 445-452.

Rojas-Grau, M. A., Avena-Bustillos, R. J., Olsen, C., Friedman, M., Henika, P. R., Martin-Belloso, O., Pan, Z. L. & McHugh, T. H. (2007). Effects of plant essential oils and oil compounds on mechanical, barrier and antimicrobial properties of alginate-apple puree edible films. Journal of Food Engineering, **81** (3), 634-641.

Roselli, M., Britti, M. S., Le Huërou-Luron, I., Marfaing, H., Zhu, W. Y. & Mengheri, E. (2007). Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic *Escherichia coli* K88 in pig intestinal cells. Toxicolology In Vitro, **21** (2), 224-229.

Roselli, M., Finamore, A., Britti, M. S., Bosi, P., Oswald, I. & Mengheri, E. (2005). Alternatives to in-feed antibiotics in pigs: evaluation of probiotics, zinc or organic acids as protective agents for the intestinal mucosa. A comparison of *in vitro* and *in vivo* results. Animal Research, **54**, 203-218.

Ruberto, G. & Baratta, M. T. (2000). Antioxidant activity of selected essential oil components in two lipid model systems. Food Chemistry, **69** (2), 167-174.

Sambrook, I. E. (1981). Studies on the flow and composition of bile in growing-pigs. Journal of the Science of Food and Agriculture, **32** (8), 781-791.

Sangster, S. A., Caldwell, J. & Smith, R. L. (1984a). Metabolism of anethole .2. Influence of dose size on the route of metabolism of *trans*-anethole in the rat and mouse. Food and Chemical Toxicology, **22** (9), 707-713.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A. & Smith, R. L. (1987). The metabolic disposition of [methoxy-C-14]-labeled trans-anethole, estragole and *p*-propylanisole in human volunteers. Xenobiotica, **17** (10), 1223-1232.

Sangster, S. A., Caldwell, J., Smith, R. L. & Farmer, P. B. (1984b). Metabolism of anethole .1. Pathways of metabolism in the rat and mouse. Food and Chemical Toxicology, **22** (9), 695-706.

Santiesteban-Lopez, A., Palou, E. & Lopez-Malo, A. (2007). Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected a(w) and pH. Journal of Applied Microbiology, **102** (2), 486-497.

Sapienza, P. P., Ikeda, G. J., Warr, P. I., Plummer, S. L., Dailey, R. E. & Lin, C. S. (1993). Tissue distribution and excretion of labelled cinnamic aldehyde following single and multiple oral administration in male fischer 344 rats. Food Chemical Toxicology, **31** (4), 253-261.

Sarkozi, S., Almassy, J., Lukacs, B., Dobrosi, N., Nagy, G. & Jona, I. (2007). Effect of natural phenol derivatives on skeletal type sarcoplasmic reticulum Ca^{2+}-ATPase and ryanodine receptor. Journal of Muscle Research and Cell Motility, **28** (2-3), 167-174.

Sasaki, K., Wada, K., Tanaka, Y., Yoshimura, T., Matuoka, K. & Anno, T. (2005). Thyme (*Thymus vulgaris* L.) leaves and its constituents increase the activities of xenobiotic-metabolizing enzymes in mouse liver. Journal of Medicinal Food, **8** (2), 184-189.

Schoevers, E. J., Van Leengoed, L. A. M. G., Verheijden, J. H. M. & Niewold, T. A. (1999). Effects of enrofloxacin on porcine phagocytic function. Antimicrobial Agents Chemotherapy, **43** (9), 2138-2143.

Schöne, F., Vetter, A., Hartung, H., Bergmann, H., Biertumpfel, A., Richter, G., Muller, S. & Breitschuh, G. (2006). Effects of essential oils from fennel (*Foeniculi aetheroleum*) and caraway (*Carvi aetheroleum*) in pigs. Journal of Animal Physiology and Animal Nutrition, **90** (11-12), 500-510.

Seyfarth, A. M., Wegener, H. C. & Frimodt-Moller, N. (1997). Antimicrobial resistance in *Salmonella enterica* subsp. *enterica* serovar Typhimurium from humans and production animals. Journal of Antimicrobial Chemotherapy, **40** (1), 67-75.

Shahverdi, A. R., Monsef-Esfahani, H. R., Tavasoli, F., Zaheri, A. & Mirjani, R. (2007). *Trans*-cinnamaldehyde from *Cinnamomum zeylanicum* bark essential oil reduces the clindamycin resistance of *Clostridium difficile in vitro*. Journal of Food Science, **72** (1), S55-S58.

Shapira, R. & Mimran, E. (2007). Isolation and characterization of *Escherichia coli* mutants exhibiting altered response to thymol. Microbial Drug Resistance-Mechanisms Epidemiology and Disease, **13** (3), 157-165.

Shapiro, S., Meier, A. & Guggenheim, B. (1994). The antimicrobial activity of essential oils and essential oil components towards oral bacteria. Oral Microbiology and Immunology, **9** (4), 202-208.

Shelef, L. A., Jyothi, E. K. & Bulgarelli, M. A. (1984). Growth of enteropathogenic and spoilage bacteria in sage-containing broth and foods. Journal of Food Science, **49** (3), 737-740.

Shimoni, E., Baasov, T., Ravid, U. & Shoham, Y. (2002). The *trans*-anethole degradation pathway in an *Arthrobacter* sp. Journal of Biological Chemistry, **277** (14), 11866-11872.

Si, W., Gong, J., Chanas, C., Cui, S., Yu, H., Caballero, C. & Friendship, R. M. (2006a). *In vitro* assessment of antimicrobial activity of carvacrol, thymol and cinnamaldehyde towards *Salmonella* serotype Typhimurium DT104: effects of pig diets and emulsification in hydrocolloids. Journal of Applied Microbiology, **101** (6), 1282-1291.

Si, W., Gong, J., Tsao, R., Zhou, T., Yu, H., Poppe, C., Johnson, R. & Du, Z. (2006b). Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. Journal of Applied Microbiology, **100**, 296-305.

Sikkema, J., De Bont, J. A. M. & Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews, **59** (2), 201-222.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T. & Arsenakis, M. (1996). Antimicrobial and cytotoxic activities of *Origanum* essential oils. Journal of Agricultural and Food Chemistry, **44**, 1202-1205.

Slamenova, D., Horvathova, E., Marsalkova, L. & Wsolova, L. (2008). Carvacrol given to rats in drinking water reduces the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H_2O_2. Neoplasma, **55**, 394 - 399.

Smith-Palmer, A., Stewart, J. & Fyfe, L. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Applied Microbiology, **26** (2), 118-122.

Smith-Palmer, A., Stewart, J. & Fyfe, L. (2001). The potential application of plant essential oils as natural food preservatives in soft cheese. Food Microbiology, **18** (4), 463-470.

Soares, P. M. G., Lima, R. F., Pires, A. D., Souza, E. P., Assreuy, A. M. S. & Criddle, D. N. (2007). Effects of anethole and structural analogues on the contractility of rat isolated aorta: involvement of voltage-dependent Ca^{2+}-channels. Life Sciences, **81** (13), 1085-1093.

Söderholm, J. D., Hedman, L., Artursson, P., Franzen, L., Larsson, J., Pantzar, N., Permert, J. & Olaison, G. (1998). Integrity and metabolism of human ileal mucosa in vitro in the Ussing chamber. Acta Physiologica Scandinavica, **162** (1), 47-56.

Sollmann, T. (1919). Experiments with carvacrol. Journal of Pharmacology and Experimental Therapeutics, **14** (3), 251-258.

Southwell, I. A., Hayes, A. J., Markham, J. & Leach, D. N. (1993). The search for optimally bioactive Australian tea tree oil. Acta Horticulturae, **334**, 256-265.

Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.-L. & Von Wright, A. (1999). Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food and Chemical Toxicology, **37**, 813-823.

Steele, R. H. (1997). Clove oil, eugenol, isoeugenol and methyol eugenol. Minnesota Letter Request 19961216. Accessed online http://legacy.library.ucsf.edu/tid/aen58d00.

Stoni, A., Zitterl-Eglseer, K., Kroismayr, A., Wetscherek, W. & Windisch, W. (2005). Ätherische Öle in der Ferkelfütterung: Effekt auf die Nährstoffverdaulichkeit und die Wiederfindung im Gewebe. In: Proceedings of the 4-BOKU Symposium Tierernährung, 27. Oktober 2005, Wien, Austria, p. 147-153.

Subash Babu, P., Prabuseenivasan, S. & Ignacimuthu, S. (2007). Cinnamaldehyde - a potential antidiabetic agent. Phytomedicine, **14** (1), 15-22.

Sutton, J. D., Sangster, S. A. & Caldwell, J. (1985). Dose-dependent variation in the disposition of eugenol in the rat. Biochemical Pharmacology, **34** (3), 465-466.

Suzuki, Y., Sugiyama, K. & Furuta, H. (1985). Eugenol-mediated superoxide generation and cyto-toxicity in Guinea-pig neutrophils. Japanese Journal of Pharmacology, **39** (3), 381-386.

Szentandrassy, N., Szentesi, P., Magyar, J., Nanasi, P. P. & Csernoch, L. (2003). Effect of thymol on kinetic properties of Ca and K currents in rat skeletal muscle. BMC Pharmacology, **3**, 1-8.

Szentandrassy, N., Szigeti, G., Szegedi, C., Sarkozi, S., Magyar, J., Banyasz, T., Csernoch, L., Kovacs, L., Nanasi, P. P. & Jona, I. (2004). Effect of thymol on calcium handling in mammalian ventricular myocardium. Life Sciences, **74** (7), 909-921.

Tadasa, K. (1977). Degradation of eugenol by a microorganism. Agricultural and Biological Chemistry, **41** (6), 925-929.

Takada, M., Agata, I., Sakamoto, M., Yagi, N. & Hayashi, N. (1979). On the metabolic detoxication of thymol in rabbit and man. Journal of Toxicological Science, **4** (4), 341-350.

Terpene Consortium (2002). Test plan and robust summaries for anethole (isomer unspecified) and *trans*-anethole. Report nr. ARZ01-14069; submitted to the EPA under the HPV Challenge Program by: The Flavor and Fragrance High Production Volume Consortia, Terpene Consortium, Washington, D.C. 20006.

Thomke, S. & Elwinger, K. (1998). Growth promotants in feeding pigs and poultry. III. Alternatives to antibiotic growth promotants. Annales de Zootechnie, **47** (245-271).

Thoroski, J., Blank, G. & Biliaderis, C. (1989). Eugenol induced-inhibition of extracellular enzyme-production by *Bacillus subtilis*. Journal of Food Protection, **52** (6), 399-403.

Trevisi, P., Merialdi, G., Mazzoni, M., Casini, L., Tittarelli, C., De Filippi, S., Minieri, L., Lalatta-Costerbosa, G. & Bosi, P. (2007). Effect of dietary addition of thymol on growth, salivary and gastric function, immune response, and excretion of *Salmonella enterica* serovar Typhimurium, in weaning pigs challenged with this microbe strain. Italian Journal of Animal Science, **6** (Suppl. 1), 374-376.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. & Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. Antimicrobial Agents and Chemotherapy, **49** (6), 2474-2478.

Tsinas, A. C., Ginnakopoulos, C. G., Papasteriades, A., Alexopoulos, C., Mavromatis, J. & Kyriakis, S. C. (1998a). Use of *Origanum* essential oils as growth promoter in pigs. In: Proceedings of the 15th IPVS Congress, Birmingham, UK, 5-9 July 1998, 3, 221.

Tsinas, A. C., Kyriakis, S. C., Bourtzi-Chatzopoulou, E., Arsenakis, M., Sarris, K., Papasteriades, A. & Lekkas, S. (1998b). Control of porcine proliferative enteropathy by in-feed application of *Origanum* essential oils. In: Proceedings of the 15th IPVS Congress, Birmingham, UK, 5-9 July 1998, 3, 106.

Turner, J. L., Dritz, S. S. & Minton, J. E. (2001). Review: alternatives to conventional antimicrobials in swine diets. The Professional Animal Scientist, **17**, 217-226.

Ultee, A. & Smid, E. J. (2001). Short communication. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. International Journal of Food Microbiology, **64**, 373-378.

Ultee, A., Bennik, M. H. J. & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology, **68** (4), 1561-1568.

Ultee, A., Kets, E. P. W. & Smid, E. J. (1999). Mechanism of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology, **65** (10), 4606-4610.

Ultee, A., Kets, E. P. W., Alberda, M., Hoekstra, F. A. & Smid, E. J. (2000a). Adaptation of the food borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology, **174**, 233-238.

Ultee, A., Slump, R. A., Steging, G. & Smid, E. J. (2000b). Antimicrobial activity of carvacrol toward Bacillus cereus on rice Journal of Food Proctection, **63** (5), 620-624.

Van Der Heyde, H. & Henderickx, H. (1963). Zur Vereinfachung der quantitativen und qualitativen Bestimmung der Bakterien unter Verwendung von 'Ringplatten'. Z. Bakteriol. I. Orig, **189**, 224-228.

Van Iersel, M. L. P. S., Ploemen, J.-P. H. T. M., Lo Bello, M., Federici, G. & van Bladeren, P. J. (1997). Interactions of *α,β*-unsaturated aldehydes and ketones with human glutathione S-transferase P1-1. Chemico-Biological Interactions, **108** (1-2), 67-78.

Van Krimpen, M. M. & Binnendijk, G. P. (2001). Ropadiar als alternatief voor een antimicrobiële groeibevorderaar in voer voor gespeende biggen. Rapport - Praktijkonderzoek Veehouderij, No. 205 14.

Van Nevel, C., Decuypere, J. A., Dierick, N. & Molly, K. (2003). The influence of *Lentinus edodes* (Shiitake mushroom) preparations on bacteriological and morphological aspects of the small intestine in piglets. Archives of Animal Nutrition, **57**, 399-412.

Van Zyl, R.L., Seatlholo, S.T., Van Vuuren, S.F. & Viljoen, A.M. (2006). The biological activities of 20 nature identical essential oil constituents. Journal of Essential Oil Research, **18**, 129-133.

Vander Jagt, D. L., Hunsaker, L. A., Vander Jagt, T. J., Gomez, M. S., Gonzales, D. M., Deck, L. M. & Royer, R. E. (1997). Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. Biochemical Pharmacology, **53** (8), 1133-1140.

Vangroenweghe, F. (2008). Antibioticumgevoeligheid van *Brachyspira* species, een stand van zaken. Varkensbedrijf, **19**, 3, 26-27

Varel, V. H. & Miller, D. L. (2004). Eugenol stimulates lactate accumulation yet inhibits volatile fatty acid production and eliminates coliform bacteria in cattle and swine waste. Journal of Applied Microbiology, **97**, 1001-1005.

Varel, V. H. (2002). Carvacrol and thymol reduce swine waste odor and pathogens: stability of oils. Current Microbiology, **44**, 38-43.

Veldhuizen, E. J. A., Creutzberg, T. O., Burt, S. A. & Haagsman, H. P. (2007). Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against *Listeria monocytogenes* in steak tartare. Journal of Food Protection, **70** (9), 2127-2132.

Veldhuizen, E. J. A., Tjeerdsma-Van Bokhoven, J. L. M., Zweijtzer, C., Burt, S. A. & Haagsman, H. P. (2006). Structural requirements for the antimicrobial activity of carvacrol. Journal of Agricultural and Food Chemistry, **54** (5), 1874-1879.

Vervaeke, I. J., Decuypere, J. A., Dierick, N. A. & Henderickx, H. K. (1979). Quantitative *in vitro* evaluation of the energy metabolism influenced by virginiamycin and spiramycin used as growth promoters in pig nutrition. Journal of Animal Science, **49** (3), 846-856.

Vichi, S., Zitterl-Eglseer, K., Jugl, M. & Franz, C. (2001). Determination of the presence of antioxidants deriving from sage and oregano extracts added to animal fat by means of assessment of the radical scavenging capacity by photochemiluminescence analysis. Nahrung, **45** (2), 101-104.

Visek, W. J. (1978). The mode of growth promotion by antibiotics. Journal of Animal Science, **46** (5), 1447-1468.

Vogt-Eisele, A. K., Keber, K., Sherkheli, M. A., Vielhaber, G., Panten, J., Gisselmann, G. & Hatt, H. (2007). Monoterpenoid agonists of TRPV3. British Journal of Pharmacology, **151** (4), 530-540.

Vokou, D. & Liotiri, S. (1999). Stimulation of soil microbial activity by essential oils. Chemoecology, **9** (1), 41-45.

Vulich, S. A., O'Riordan, E. & Hanrahan, J. P. (1991). Use of n-alkanes for the estimation of herbage intake in sheep: accuracy and precision of the estimates. Journal of Agricultural Science, **166**, 319-323.

Wald, C. (2002). Untersuchungen zur Wirksamkeit verschiedener ätherischer Öle im Futter von Aufzuchtferkeln und Broilern. Dissertation zur Erlangung des akademischen Grades doctor agriculturarum, Martin-Luther-Üniversität, Halle-Wittenberg, 109p.

Wald, C., Kluth, H. & Rodehutscord, M. (2001). Effect of different essential oils on the growth performance of piglets. Proceedings of the Society for Nutritrion and Physiology, **10**, 156.

Walsh, S. E., Maillard, J. Y., Russell, A. D., Catrenich, C. E., Charbonneau, D. L. & Bartolo, R. G. (2003). Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria. Journal of Applied Microbiology, **94** (2), 240-247.

Walter, B. M. & Bilkei, G. (2004). Immunostimulatory effect of dietary oregano etheric oils on lymphocytes from growth-retarted, low weight growing-finishing pigs and productivity. Tijdschrift voor Diergeneeskunde, **129**, 178-181.

Wells, C. L., van de Westerlo, E. M. A., Jechorek, R. P., Haines, H. M. & Erlandsen, S. L. (1998). Cytochalasin-induced actin disruption of polarized enterocytes can augment internalization of bacteria. Infection and Immunology, **66** (6), 2410-2419.

Wells, J. E. & Varel, V. H. (2008). Viability of zoonotic pathogens *Escherichia coli* and *Salmonella* in swine manure slurries with and without a urease inhibitor and thymol. Letters in Applied Microbiology, **46** (4), 477-482.

Wendakoon, C. N. & Sakaguchi, M. (1995). Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. Journal of Food Protection, **58** (3), 280-283.

Wenk, C. (2003). Growth-promotor alternatives after the ban on antibiotics. Pig News and Information, **24** (1), 11-16.

Westendarp, H. (2005). Essential oils in nutrition of poultry, swine and ruminants. Deutsche Tierarztliche Wochenschrift, **112** (10), 375-380.

Westendarp, H., Klaus, P., Halle, I., Morlein, D., Henning, M. & Kohler, P. (2006). Effect of carvacrol, gamma-terpinene and p-cymene-7-ol in broiler feed on growth traits and N-metabolism. Landbauforschung Volkenrode, **56** (3-4), 149-157.

Windisch, W. M., Schedle, K., Plitzner, C. & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science, **86**, E140-E148.

Wong, S. Y. Y., Grant, I. R., Friedman, M., Elliott, C. T. & Situ, C. (2008). Antibacterial activities of naturally occurring compounds against *Mycobacterium avium* subsp. paratuberculosis. Applied Environmental Microbiology, **74** (19), 5986-5990.

Wu, S.-J., Ng, L.-T. & Lin, C.-C. (2004). Effects of vitamin E on the cinnamaldehyde-induced apoptotic mechanism in human PLC/PRF/5 cells. Clinical and Experimental Pharmacology and Physiology, **31**, 770-776.

Xu, H. X., Delling, M., Jun, J. C. & Clapham, D. E. (2006). Oregano, thyme and clove-derived flavors and skin sensitizers activate specific TRP channels. Nature Neuroscience, **9** (5), 628-635.

Xu, J., Zhou, F., Ji, B. P., Pei, R. S. & Xu, N. (2008). The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. Letters in Applied Microbiology, **47** (3), 174-179.

Yamazaki, K., Yamamoto, T., Kawai, Y. & Inoue, N. (2004). Enhancement of antilisterial activity of essential oil constituents by nisin and diglycerol fatty acid ester. Food Microbiology, **21**, 283-289.

Yanaga, A., Goto, H., Nakagawa, T., Hikiami, H., Shibahara, N. & Shimada, Y. (2006). Cinnamaldehyde induces endothelium-dependent and -independent vasorelaxant action on isolated rat aorta. Biological & Pharmaceutical Bulletin, **29** (12), 2415-2418.

Yatzidis, H. (1974). New method for direct determination of true creatinine. Clinical Chemistry, **20**, 1131-1134.

Youdim, K. A. & Deans, S. G. (2000). Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain. British Journal of Nutrition, **83**, 87-93.

Youdim, K. A. & Deans, S. G. (2002). The antioxidant properties of thyme (*Thymus zygis* L.) essential oil: an inhibitor of lipid peroxidation and a free radical scavenger. Journal of Essential Oil Research, **14**, 210-215.

Youn, H. S., Lee, J. K., Choi, Y. J., Saitoh, S. I., Miyake, K., Hwang, D. H. & Lee, J. Y. (2008). Cinnamaldehyde suppresses toll-like receptor 4 activation mediated through the inhibition of receptor oligomerization. Biochemical Pharmacology, **75** (2), 494-502.

Yuan, J., Bucher, J. R., Goehl, T. J., Dieter, M. P. & Jameson, C. W. (1992). Quantitation of cinnamaldehyde and cinnamic acid in blood by HPLC. Journal of Analytical Toxicology, **16**, 359-362.

Yuan, J., Dieter, M. P., Bucher, J. R. & Jameson, C. W. (1993). Application of micro-encapsulation for toxicology studies. III. Bioavailability of microencapsulated cinnamaldehyde. Fundamental and Applied Toxicology, **20** (1), 83-87.

Zayed, G., Roos, Y.H., 2004. Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. Process Biochemistry, **39**, 1081-1086.

Zhou, F., Ji, B. P., Zhang, H., Jiang, H., Yang, Z. W., Li, J. J., Li, J. H. & Yan, W. J. (2007a). The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella* typhimurium. Journal of Food Safety, **27** (2), 124-133.

Zhou, F., Ji, B. P., Zhang, H., Jiang, H., Yang, Z. W., Li, J. J., Li, J. H., Ren, Y. L. & Yan, W. J. (2007b). Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella* typhimurium. Journal of Food Protection, **70** (7), 1704-1709.

Zitterl-Eglseer, K., Wetscherek, W., Stoni, A., Kroismayr, A. & Windisch, W. (2007). Detection of essential oil compounds in different animal tissues after feeding a phytogenic feed additive to pigs. In: Proceedings of the 55th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Graz, Austria September 2–6, 2007, Chairman: Prof. Dr. Rudolf Bauer. Planta Medica, **73** (9), 919.

CURRICULUM VITAE

Joris Michiels werd geboren te Oostende op 29 juni 1972. In 1995 studeerde hij met grote onderscheiding af aan de Universiteit Gent als bio-ingenieur in de landbouwkunde. Sinds februari 2004 is hij als onderzoeksassistent verbonden aan de Vakgroep Dierlijke Productie van de Hogeschool Gent. In samenwerking met de Vakgroep Dierlijke Productie van de Universiteit Gent, werkte hij gedurende deze periode aan dit doctoraal proefschrift. Aan de Hogeschool Gent, stond hij in voor de oefeningen van diverse opleidingsonderdelen in de opleidingen Bachelor en Master in de Biowetenschappen en begeleidde hij meerdere studenten bij het uitvoeren van hun masterscriptie. Hij is auteur en co-auteur van verscheidene publicaties in peer-reviewed wetenschappelijke tijdschriften. Daarnaast verzorgde hij diverse voordrachten en postervoorstellingen op nationale en internationale workshops en symposia.

PUBLICATIONS

Peer reviewed A1-publications

Michiels, J., Missotten, J. A. M., Van Hoorick, A., Ovyn, A. Fremaut, D., De Smet, S. & Dierick, N. A. (revised version submitted). Effect of dose and formulation of carvacrol and thymol on gut bacteria and gut function in piglets after weaning. Animal.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (submitted). Thymol and *trans*-cinnamaldehyde reduce active nutrient absorption and stimulated chloride secretion in the pig jejunal Ussing chamber model. Livestock Science.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D., Maene, P. & De Smet, S. (2008). *In vitro* degradation and *in vivo* passage kinetics of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde along the gastrointestinal tract of piglets. Journal of the Science of Food and Agriculture, **88**, 2371-2381.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. A. (2007). *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. Livestock Science, **109**, 157-160.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. A. (in press). *In vitro* characterisation of the antimicrobial activity of selected essential oil components and binary combinations against the pig gut flora. Animal Feed Science and Technology, doi:10.1016/j.anifeedsci.2009.01.004.

Missotten, J. A. M., Goris, J., Michiels, J., Van Coillie, E., Herman, L., De Smet, S., Dierick, N. A. & Heyndrickx, M. (2009). Screening of isolated lactic acid bacteria as potential beneficial strains for fermented liquid pig feed production. Animal Feed Science and Technology, **150**, 122-138.

Missotten, J., Michiels, J., Goris, J., Herman, L., Heyndrickx, M., De Smet, S. & Dierick, N. A. (2007). Screening of 2 probiotic products for use in fermented liquid feed. Livestock Science, **108**, 232-235.

Missotten, J. A. M., Michiels, J., Willems, W., Ovyn, A., De Smet, S. & Dierick, N. A. (submitted). Effect of fermented liquid feed on morpho-histological parameters in the piglet gut. Livestock Science.

A2-publications

Dierick, N., Michiels, J. & Van Nevel, C. (2004). Effect of medium chain fatty acids and benzoic acid, as alternative for antibiotics, on growth and some gut parameters in piglets Communications in Agricultural and Applied Biological Sciences, **69** (2), 187-190.

Michiels, J., Maene, P., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (2006). Gas-Chromatographic method for quantifying carvacrol, thymol, terpinen-4-ol, *trans*-anethole, eugenol and *trans*-cinnamaldehyde in media simulating pig gut conditons. Communications in Agricultural and Applied Biological Sciences, **71** (1), 223-226.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (2005). Antimicrobial activity of selected phytochemicals against the pig gut flora. Communications in Agricultural and Applied Biological Sciences, **70** (2), 191-194.

Missotten, J., Michiels, J., Goris, J., Herman, L., Heynderickx, M., De Smet, S. & Dierick, N. A. (2006). Fermented liquid feed for weaned piglets. Communications in Agricultural and Applied Biological Sciences, **71** (1), 227-230.

A4-publications

Fremaut, D. & Michiels, J. (2007). Een lupinenmenu voor braadkippen. Pluimvee, mei 2007, 11-12.

Fremaut, D., D'Hooghe, K. & Michiels, J. (2005). Koolzaadkoek in de diervoeding. Landbouw & Techniek, **03** (11 februari 2005), 38-40.

Fremaut, D., Ghekiere, D. & Michiels, J. (2005). Eiwitten, een noodzakelijk kwaad in het voeder. Landbouw & Techniek, **12** (24 juni 2005), 15-17.

Fremaut, D., Michiels, J. & Missotten, J. (2006). Antibioticatheorie wankelt. De Molenaar, **22** (10 november 2006), 10-12.

C1-publications

Dierick, N., Michiels, J. & Van Nevel, C. (2004). Effect of medium chain fatty acids and benzoic acid, as alternative for antibiotics, on growth and some gut parameters in piglets. In Proceedings of the 3rd AFAC Workshop on Alternatives to Feed Antibiotics and Anticoccidials In the Pig and Poultry Meat Production, September 19th-20th 2004, Aarhus, Denmark, 4p.

Fremaut, D., Michiels, J., De Smet, S., Raes, K., Van Oeckel, M. & Warnants, N. (2005). Toepassing van omega-3 vetzuren in de varkensvoeding: effect op vleeskwaliteit en diergezondheid. Studiedag 'Omega-3 vetzuren en CLA in de landbouw: perspectieven voor boer en consument', 9 maart 2005, Gent, 6p.

Fremaut, D., Michiels, J., Wullepit, N. & Nollet, L. (2008). Effect of Yea-Sacc on the persistency of the milk production in late lactation of healthy and high performing dairy cattle on a high fiber feed. Alltech's European Lecture Tour, February 11th-29th 2008, Zwijnaarde.

Michiels, J. (2006). Inzichten en perspectieven voor botanische stoffen met antimicrobiële werking in de varkensvoeding. Studiedag 'Alternatieven voor voederantibiotica: zoötechnie en voedselveiligheid verenigd?', 7 september 2006, Gent, 37p.

Michiels, J. M., L., Ringel, J., Dierick, N. A. & De Smet, S. (2009). Effect of feed supplementation with guanidinoacetic acid (GAA) on broiler performance and carcass and meat characteristics. 34th Animal Nutrition Research Forum, April 3rd 2009, Merelbeke, 59-60.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (2005). *In vitro* effect of botanicals on gut flora of pigs. 30ste Studiedag van de Nederlandstalige Voedingsonderzoekers, 8 april 2005, Merelbeke, 67-68.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D. & De Smet, S. (2005). Modifying gut flora in pigs by phytochemicals. In Proceedings of the 10th Symposium Vitamins and Additives, September 28th-29th 2005, Friedrich Schiller University Jena, Jena, Germany, 230-235.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. (2007). Fast intestinal absorption of free carvacrol and thymol compromises antibacterial effects against the pig gut flora. In Final Programme Book of Abstracts of the 38th International Symposium on Essential Oils, September 9th-12th 2007, Graz, Austria, p57.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. (2009). Effect of dose of thymol and flavours on palatability in a choice-feeding study with piglets. 34th Animal Nutrition Research Forum, April 3rd 2009, Merelbeke, 41-42.

Missotten, J. A. M., Willems, W., Michiels, J., De Smet, S. & Dierick, N. A. (2008). Effect of fermented liquid feed on the performance of weaned piglets. In Book of Abstracts of the 59th Annual Meeting of EAAP, August 24th-27th 2008, Vilnius, Lithuania, p216.

Missotten, J., Michiels, J., Goris, J., Herman, L., Heynderickx, M., De Smet, S. & Dierick, N. (2006). Fermented liquid feed for weaning piglets. 31ste Studiedag van de Nederlandstalige Voedingsonderzoekers, 7 april 2006, Rotterdam, Nederland, 16-17.

Missotten, J. A. M., Michiels, J., De Smet, S. & Dierick, N. (2009). Effect of Sepiolite on sedimentation of the solids in (fermented) liquid feed. 34th Animal Nutrition Research Forum, April 3rd 2009,Merelbeke, 47-48.

Van Hoorick, A., Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N. (2008). Effect of dose and formulation of thymol on gut bacteria and indices of gut functionality of weaned piglets. 33ste Studiedag van de Nederlandstalige Voedingsonderzoekers, 25 april 2008, Wageningen, Nederland, p39.

Willems, W., Missotten, J. A. M., Michiels, J., De Smet, S. & Dierick, N. (2008). Fermented liquid feed, inoculated with a probiotic strain, for weaning piglets. 33ste Studiedag van de Nederlandstalige Voedingsonderzoekers, 25 april 2008, Wageningen, Nederland, p61.

CONFERENCES, WORKSHOPS, SEMINARS

2004

- 3rd AFAC Workshop on Alternatives to Feed Antibiotics and Anticoccidials In the Pig and Poultry Meat Production, September 19th-20th, Aarhus, Denmark. Effect of medium chain fatty acids and benzoic acid, as alternative for antibiotics, on growth and some gut parameters in piglets. *Poster presentation*

- 10th PhD Symposium on Applied Biological Sciences; 29 september, Gent. Effect of medium chain fatty acids and benzoic acid, as alternative for antibiotics, on growth and some gut parameters in piglets. *Poster presentation*

2005

- 31ste Studiedag van de Nederlandstalige Voedingsonderzoekers, 8 april 2005, Merelbeke. *In vitro* effect of botanicals on gut flora of pigs. *Poster presentation*

- 10th Symposium Vitamins and Additives, September 28th-29th, Friedrich Schiller University Jena, Jena, Germany. Modifying gut flora in pigs by phytochemicals. *Oral presentation*

- 11th PhD Symposium on Applied Biological Sciences; 6 oktober, Leuven. Antimicrobial activity of selected phytochemicals against the pig gut flora. *Poster presentation*

2006

- 10th International Symposium on Digestive Physiology in Pigs, May 24th-27th, Vejle, Denmark. *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. *Poster presentation*

- Studiedag 'Alternatieven voor voederantibiotica: zoötechnie en voedselveiligheid verenigd?', 7 september, Gent. Inzichten en perspectieven voor botanische stoffen met antimicrobiële werking in de varkensvoeding. *Oral presentation*

- 12th PhD Symposium on Applied Biological Sciences; 21 september, Gent. Gas-Chromatographic method for quantifying carvacrol, thymol, terpinen-4-ol, *trans*-anethole, eugenol and *trans*-cinnamaldehyde in media simulating pig gut conditons. *Poster presentation*

2007

- 38th International Symposium on Essential Oils, September 9th-12th, Graz, Austria. Fast intestinal absorption of free carvacrol and thymol compromises antibacterial effects against the pig gut flora. *Oral presentation*

2009

- 34th Animal Nutrition Research Forum, April 3rd, Melle. Effect of feed supplementation with guanidinoacetic acid (GAA) on broiler performance and carcass and meat characteristics. *Poster presentation*

- 34th Animal Nutrition Research Forum, April 3rd, Melle. Effect of dose of thymol and flavours on palatability in a choice-feeding study with piglets. *Oral presentation*

DANKWOORD

Toen ik op de Hogeschool Gent terecht kwam wist ik eigenlijk niet wat me te wachten stond. Op het sollicitatie gesprek was het duidelijk geworden dat het onderzoek iets met varkens zou zijn. Echte affectie met die dieren had ik niet, mijn ouders hadden melkkoeien en die varkens die stinken altijd. De eerste gesprekken met mijn promotoren verliepen vlot, en ze kwamen met het idee op de proppen om te onderzoeken wat essentiële oliën zouden kunnen betekenen voor biggen. Natuurlijk spitste ik mijn oren want essentiële oliën dat zijn aroma's en aangename geuren en al vlug legde ik de link met die varkens. Achteraf bleek natuurlijk dat ze daar andere bedoelingen mee hadden.

Ik bleef niet bij de pakken zitten en ging te rade bij echte specialisten ter zake. In leerde Philippe Gerard van de Primrose Academy kennen. Philippe, een echte merci. Je hebt me ingeleid in de fascinerende wereld van de fytotherapie, aromatherapie … Veel suggesties en ideeën voor onderzoek heb ik bij jou opgedaan, en de cursussen die ik bij je volgde waren echt boeiend. De eerste keer dat we een essentiële olie mochten extraheren in je labo vergeet ik nooit.

Zo was mijn ontdekkingstocht gestart, en beetje bij beetje leerde ik meer over onze varkens. Heel veel kon ik opsteken van mijn promotoren: Noël Dierick, Stefaan De Smet en Dirk Fremaut. Noël, je hebt me van dichtbij begeleid in het onderzoek. Zo vele malen stond ik aan je bureau met vragen en twijfels over het proefprotocol en steeds hielp je me vooruit. Je encyclopedische kennis over verteringsfysiologie, grondstoffen en additieven is onuitgegeven. Je kritische kijk op mijn onderzoek en resultaten en het minitieus doornemen van de manuscripten waardeer ik ten zeerste. Ik wil je hiervoor oprecht van harte bedanken. Stefaan, je was een aandachtige toeschouwer van het werk. Bij besprekingen had je altijd een klare kijk op de zaak en kwam je met verrassende opmerkingen uit de hoek. Bovendien gaf je jou mening eerlijk te kennen, zowel als het goed of slecht was. Bedankt voor de leerrijke samenwerking de voorbije jaren. Dikwijls was Dirk de eerste persoon die ik op het werk tegenkwam. We hebben dan ook veel gepraat, pittige verhalen verteld, gediscussieerd en over nieuwe ideeën nagedacht. Je informeerde altijd hoe het ervoor stond en dit was steeds een stimulans om verder te doen. Bedankt Dirk. Mijn promotoren wil ik ook bedanken voor de nodige vrijheid die ze me gaven bij het uitvoeren van mijn taken; zowel bij het doctoraatsonderzoek als bij de lesopdrachten. Ik wil hierbij ook nog speciaal Chris Van Nevel bedanken. Bedankt voor je hulp en suggesties bij het onderzoek en de levenswijsheid die jij ons, jonge onderzoekers altijd meegaf.

I also would like to thank sincerely all the members of the examination committee for reading the manuscript and for all suggestions to improve my thesis. Especially, the attendance of the researchers from abroad is greatly appreciated.

De voorbije jaren waren een continu zwerven tussen Lanupro en BIOT. De experimenten gingen door op Lanupro, in het labo 'beneden'. Daar heb ik een fantastische tijd gehad, wat alleen maar mogelijk was door de collega's daar; Cyriel, Anneke, Annick en Erwin. Bedankt voor jullie eindeloze inzet en hulp bij alle labowerk en proeven, en bij de opkuis die ik liet staan. Het ene moment was het een geoliede machine, het andere moment was er tijd voor gebrul en gelach. Het was bijzonder aangenaam om jullie één voor één beter te leren kennen. Cyriel, je verhalen over je fietstochten waren aanstekelijk (natuurlijk was ik altijd jaloers). Bedankt voor alle praktische hulp. Ann, vele protocols heb ik met jou doorgenomen en veelal nam jij de leiding bij de slachtproeven. En je had altijd tijd voor een babbel. Merci voor de aangename tijd in het labo (en nu ben ik echt serieus!). Annick, merci voor de vele analyses, de uitplatingen en de oprechte babbels tussendoor. Tenslotte is er nog Erwin, die steeds de voeders maakte, voor de dieren zorgde, maar eigenlijk een man van alle werk is. Merci voor al je werk, en natuurlijk ook de leute, onvergetelijk zijn die sneeuwman en het strippen.

De bureau op Lanupro moest ik delen met een andere Joris, wat soms tot hilarische situaties leidde. Joris, in het begin was je de cel-en-genner die het wel zou weten. Al gauw moest ik mijn mening herzien. We leerden elkaar gaandeweg beter kennen en respecteren. We hadden ook veel dezelfde interesses (hell yeah). Je was een supertoffe kameraad. Aan je enthousiasme heb ik me heel dikwijls kunnen optrekken. Bedankt voor de vele wetenschappelijke discussies en de hulp in het labo. Op moeilijke momenten konden we over alles praten. Een grote merci voor al die jaren en de vriendschap; merci ook aan Elke.

Uiteraard wil ik ook al de andere collega's van Lanupro en BIOT die op één of andere manier mij geholpen hebben bedanken. Waardering voor het werk van Charlotte. Jouw tabelletje met richtlijnen en bijhorend tijdschema voor wie in de laatste rechte lijn van zijn lange tocht is belandt geeft me veel gemoedsrust bezorgd. En voorts al degenen die ik leerde kennen op studiedagen of bezoeken en mijn blik op essentiële oliën en varkens verruimden of mijn praktisch hielpen bij de voorbereiding van de proeven.

Dank aan Paul Vandecasteele, alias "*the simplisitie of the measerment is its bjoetie, de result is de interpreteisjen of tat simpel measerment and noting seems to be wat it reely is*" voor de vele uren,

belangeloze hulp bij de Ussing kamers. Het was zwoegen en hard nadenken om het telkens weer allemaal in orde te krijgen. We hebben beide veel bijgeleerd en je was een bijzondere verschijning in ons labo. Merci dat ik op jou kon rekenen.

Een pluim voor Luc Decombel, je hebt me dikwijls uit de nood geholpen met statistische problemen, ook na de uren bij jullie thuis. Het gedoe met de gaschromatograaf was niet mogelijk geweest zonder de hulp van Peter Maene en Yvan De Bock. Problemen hebben we genoeg gehad; de gastoevoer die stilviel, de naald die brak, de septa van de vialtjes die niet geschikt bleken te zijn …. Altijd heb je meegezocht naar oplossingen, en met een gepaste dosis voor humor kon je alles relativeren. Bedankt voor die steun.

Een dikke merci ook aan alle toffe collega's op Lanupro en BIOT voor de veel te lange koffiepauzes, voor de babbels over de promoties van de macro en nonsens, de roddels, de traktaties, de geanimeerde recepties, de natte sportdagen, maar vooral, vooral voor de steun, de interesse en de vriendschap. Katrien en Nicolas, bedankt om van onze bureau een leuke plek te maken! Ik wil graag ook alle collega's die nog onderweg zijn, veel moed toewensen.

Mijn ouders wil ik bedanken voor de kansen die ze me gaven, voor de interesse en steun.

Sabine, het meeste heb ik aan jou te danken. Zonder je geduld, begrip en steun was het nooit gelukt. Als het teveel werd voor mij, trokken we er samen op uit en je zorgde voor rust. Oef, waarschijnlijk ben je net zo content als ik dat het erop zit. Bedankt voor al die jaren samen, merci voor alles.

Joris
7 mei 2009

“See my dreams; they're not like anyone's, anyone's” (PL X)

“Could there be, something that your world depends on in me...
Could it be, nothing that you learned from ash and debris?” (PL IR)